THE ENZYMIC 11β-HYDROXYLATION OF STEROIDS.

A Thesis presented for the degree

of

DOCTOR OF PHILOSOPHY

by

Alexander C. Brownie, B.Sc.,

Department of Biochemistry,

University of Edinburgh.

1955.

ACKNOWLEDGEMENTS

The author wishes to record his appreciation of the advice and encouragement of Professor G.F. Marrian, F.R.S. and Dr J.K. Grant, under whose supervision these investigations were carried out.

He is also indebted to Dr C.L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire, for a gift of 11-deoxycorticosterone acetate. The 11β-hydroxyprogesterone was kindly given by Dr R. Levine, The Upjohn Company, Kalamazoo, Michigan, U.S.A.

Thanks are also due to Dr J.W. Minnis who carried out the micro-analyses; to Dr E. Gilchrist, Clinical Laboratory, Royal Infirmary, Edinburgh, for nitrogen determinations; to Mrs S.M. Atherden, who performed the formaldehyde determinations; and to Mr D.W. Davidson and Mr R. Bayne for skilled technical assistance.

The co-operation of the manager and staff of the Offals Department, Wholesale Meat Marketing Association, Edinburgh, in the collection of fresh adrenal glands was of great value.

CONTENTS

GENERAL INTRODUCTION

GENERAL INTRODUCTION

It is always of interest to recall the events leading to the investigation of any research problem. In this case the chief event was the 2nd International Congress of Biochemistry held in Paris in 1952. Dr Grant of this Department, who at that time was very interested in the effects of steroid hormones on reactions of the citric acid cycle and on energy metabolism in general, in discussion with Dr Pincus of the Worcester Foundation for Experimental Biology, suggested a possible role of citric acid cycle intermediates in steroid 11β-hydroxylation catalysed by the adrenal gland. Several workers (Kahnt & Wettstein, 1951; Hayano, Dorfman & Yamada, 1951) found that steroid 11β-hydroxylation catalysed by adrenal homogenates requires the presence of citric acid cycle intermediates. Dr Grant suggested that oxidation of these citric acid cycle intermediates was required to provide energy, perhaps in the form of adenosine triphosphate (ATP), for the 11β-hydroxylation reaction. He suggested trying to inhibit 11β-hydroxylation using a reagent such as 2:4-dinitrophenol which is known to uncouple oxidative phosphorylation (Loomis & Lipmann, 1948).

The appreciation of the profound influence of steroid hormones of the adrenal gland on metabolism, and an interest in the biogenesis of adrenocortical hormones, stimulated many workers to investigate enzymic hydroxylation of adrenal steroids. The investigation of the biosynthesis of adrenocortical hormones has been of particular interest as some quite positive results have been obtained, in contrast to the investigation of the effects of these hormones on metabolism. In steroid hydroxylation studies involved in the investigation of the biosynthetic mechanism, one important line of approach has been to present known steroids to the adrenal gland using either perfusion of adrenals or incubations with slices, breis, homogenates or cell fractions. This type of investigation has yielded information regarding the reactions of which the adrenal is capable, as well as the nature of the compounds upon which such transformations can be carried out *in vitro*. Of course, the results obtained do not necessarily indicate the *in vivo* process of adrenocortical hormone synthesis.

Hechter *et al.* (1949) showed that perfusion of DOC through an adrenal gland yielded a steroid more

biologically active in the glycogen deposition test. Corticosterone was the sole metabolite and its production was not affected by ACTH. Similarly, 17α-hydroxy-11-deoxycorticosterone acetate could be transformed to 17α-hydroxycorticosterone (Hechter et al., 1950) again by 11β-hydroxylation. Perfusion of progesterone led to the isolation of 17α-hydroxy-progesterone, 11β-hydroxyprogesterone, corticosterone and 17α-hydroxycorticosterone, indicating that the gland could also introduce 17 and 21 hydroxyl groups into a preformed steroid.

It has been shown by many workers that the intact gland is not required for these hydroxylation reactions. Thus Hayano, Dorfman & Prins (1949) and Hayano, Dorfman & Yamada (1951) incubated DOC with adrenal slices, minces and homogenates and observed an increased glycogenic activity in the products which they ascribed to the formation of 11β-hydroxy steroids. Fumarate, ATP and Mg ions stimulated the reaction when homogenates were used. These preparations could more efficiently metabolize DOC glucoside than DOC, presumably due to its greater solubility. Savard, Green & Lewis (1950) obtained evidence for the formation of 11β-hydroxy derivatives from DOC and 17α-hydroxy-11-deoxy-

corticosterone on incubation with adrenal homogenates without added fumarate or other cofactors. McGinty, Smith, Wilson & Worrel (1950) isolated 17α-hydroxy-corticosterone in 50% yield as a transformation product of 17α-hydroxy-11-deoxycorticosterone incubated with adrenal homogenates supplemented with glucose and fumarate. On incubation of 100 mg. DOC with hog adrenal breis containing nicotinamide, Haines (1952) isolated 26.5 mg. corticosterone, 0.4 mg. 17α-hydroxycorticosterone, and 0.2 mg. 11-dehydro-corticosterone. Incubation of 17α-hydroxy-11-deoxycorticosterone gave a 39% yield of cortisone and 17α-hydroxycorticosterone, mainly 17α-hydroxy-corticosterone. Kahnt & Wettstein (1951) found that ATP or adenylic acid could replace fumarate as a cofactor in the 11β-hydroxylation reaction. Nicotinamide included in the homogenate stimulated the reaction suggesting a requirement for pyridine nucleotides. Glucose, Mg ions and phosphate were without effect on the 11β-hydroxylation of DOC or 17α-hydroxy-11-deoxycorticosterone. Kahnt & Wettstein found that every member of the citric acid cycle and substances such as glutamate, aspartate and lactate, which are catabolized via the citric acid cycle, were active in allowing 11β-hydroxylation to

occur. These authors suggested that processes of oxidative phosphorylation may be involved, this being an important characteristic of the citric acid cycle. They also suggested that the steroid 11β-hydroxylation may be catalysed by enzymes of Green's 'cyclophorase' system (Green, Loomis & Auerbach, 1948). 'Cyclophorase' preparations, however, are not cytoplasmically homogeneous (Harman, 1950). These preparations contain intact cells, nuclei and mitochondria. Credit for showing that 11β-hydroxylating enzymes are associated with adrenal cell particles, which by their method of preparation were mainly mitochondria, is due to Sweat (1951). Sweat homogenized adrenal tissue in isotonic sucrose and fractionated the homogenate following the method of Schneider & Hogeboom (1950). He reported that all the enzymic activity required for the conversion of 17α-hydroxy-11-deoxycorticosterone to 17α-hydroxy-corticosterone was situated in the mitochondrial fraction. No activity was found in the nuclear, microsomal or supernatant fractions. He isolated crystalline 17α-hydroxycorticosterone after incubation of 17α-hydroxy-11-deoxycorticosterone with adrenal-cell particles in a medium supplemented with glucose, fumarate and Mg ions. Addition of ATP

did not enhance the conversion.

In further studies, Hayano & Dorfman (1953) extended their studies of the 11β-hydroxylation of DOC using a so-called purified ox-adrenal homogenate residue. Whole adrenals were homogenized in saline in a Waring Blendor and centrifuged at 5000 g for 25 minutes. The residue obtained was twice washed with saline, each time being recentrifuged at 5000 g. DOC was converted into corticosterone but other unidentified trace products were also detected. The authors claim to have demonstrated the 'specificity and absolute necessity for fumarate and magnesium ions and the stimulating capacity of ATP and diphosphopyridine nucleotide (DPN)'. They suggest that fumarate may play some part in an energy-yielding system for the regeneration of ATP, or that it may function as a hydrogen acceptor in the steroid 11β-hydroxylation reaction.

Much of the previous work on enzymic hydroxylation of steroids is open to the criticisms that enzyme preparations and methods of steroid analysis have not been satisfactory. These criticisms will be dealt with more fully in the discussion. Probably on account of these features of

earlier investigations the cofactor requirements for the enzymic *in vitro* 11β-hydroxylation of steroids were not known. Previous work has pointed to certain cofactors being necessary without indicating in any way the reason for such requirement.

With regard to the mechanism of 11β-hydroxylation, Hayano & Dorfman (1953) have suggested the formation of an unsaturated intermediate, which adds on the elements of water. Levy *et al.* (1953) propose a simpler mechanism involving formation of a steroid free radical. More recently, Miescher *et al.* (1954) have obtained 11β-hydroxylation of 9:11-anhydro-17α-hydroxycorticosterone in 20% yield using adrenal homogenates. On the other hand, Hayano & Dorfman (1954a) in an experiment with D_2O showed that no deuterium entered a stable position in the steroid molecule in the course of 11β-hydroxylation.

The authors say that these facts would tend to exclude the possibility of any biosynthetic mechanism involving the direct addition of water. Using 9:11-anhydrocorticosterone and 9:11-anhydro-17α-hydroxycorticosterone these workers obtained no more than 5-10% of a more polar product using incubation conditions which would convert DOC or 17α-hydroxy-11-deoxycorticosterone to their 11β-hydroxy derivatives in 95-100% yield. The possibility of direct hydration of an unsaturated

steroid would appear doubtful.

With a view to studying these outstanding problems an investigation of the *in vitro* 11β-hydroxylation of DOC was undertaken. The chief aim of this work being the attempted elucidation of the role of cofactors like fumarate in the 11β-hydroxylation reaction. Advantage has been taken of a specific, sensitive, and accurate method for the determination of progesterone and related steroids in tissue preparations, which has been developed in this Department (Taylor, 1954).

GENERAL METHODS AND MATERIALS

a) Experimental animals.

Adrenal glands were obtained from the abattoir from oxen killed by stunning and exsanguination. These glands were obtained within 20 minutes of death of the animal, stripped of fat and connective tissue and stored in a deep-freeze cabinet until collected, packed in crushed ice, for transport to the laboratory. The manner of collection of the adrenal glands was undoubtedly important, as on occasion glands were supplied which had been lying on the slaughter-house floor for several hours. This occurred in the early days of this work, and on these occasions the enzymic activity of the glands was poor. However, after this initial stage, glands have been collected very efficiently and conscientiously by Mr J. Stanton at Gorgie Abattoir. In general, the adrenal glands used were obtained from oxen killed between 7.30 and 8 a.m. The glands were collected at 8.15 a.m. and the experiments started at about 8.45 a.m. In experiments involving adrenocortical mitochondria incubations were normally begun within 3 hours of the death of the animal.

b) Ox-adrenocortical preparations.

1) Separation of ox-adrenocortical tissue.

Adrenal glands were first carefully stripped of fat and connective tissue, then slit through the middle lengthways. This operation showed clearly the medullary and cortical tissues, the light coloured medulla in the centre with the red cortical tissue surrounding it. It was then very easy to scrape off the medulla, leaving the cortical tissue and capsule. The cortex was then separated from the capsule using a scalpel. These operations were conveniently carried out on a porcelain tile kept cool on a block of ice. The medullary tissue was discarded and the cortical tissue stored in a Petri dish standing on ice.

2) Preparation of adrenocortical homogenates.

In preliminary investigations it was found that cortical tissue was not easily homogenized as compared with liver. Dr Grant, in this Department, has introduced the use of homogenizers similar to the all-glass homogenizers used by Potter & Elvehjem (1936) having a glass tube of accurately uniform internal diameter and a well fitting Nylon pestle, grooved longitudinally at three points.

Brendler (1951) using a similar homogenizer found that the homogenates prepared in this apparatus were more uniform, with fewer intact cells, than those prepared in the all-glass homogenizer. This homogenizer has the added advantage that it shows no apparent sign of wear in use over long periods whereas the ground surfaces of the all-glass homogenizer wear rapidly.

With liver tissue, uniform and efficient homogenization is obtained with the Nylon pestle homogenizer. However, it was found that it was impossible to homogenize adrenocortical tissue in this homogenizer. To overcome this difficulty the tissue (15 g.) was finely ground in 45 ml. isotonic (0.25M) sucrose, using a loosely fitting pestle of an all-glass homogenizer. Grinding of the tissue was continued in an apparatus of similar design using a Nylon pestle. In general the cortical tissue was homogenized for 1 minute in each homogenizer. However, even this lengthy procedure does not appear to give as efficient homogenization of the adrenocortical cells as with liver. The percentage of intact cells is higher than with liver, resulting in a lower yield of mitochondria. It has

been found more convenient to use a small Latapie mincer in the cold room at 0° for the preliminary disintegration of the adrenocortical tissue. The introduction of this step, prior to homogenization in the all-glass and Nylon homogenizers has led to smoother and more efficient homogenization.

3) Preparation of ox-adrenocortical mitochondria.

The final homogenate from 15 g. cortical tissue was diluted to 90 ml. with isotonic sucrose. Nuclei and intact cells were removed by centrifuging for 10 minutes at 700 g. The supernatant was carefully removed and centrifuged twice at 5000 g for 10 minutes to sediment mitochondria. Care was taken to obtain as pure a mitochondrial preparation as possible, rather than a quantitative yield of these particles. The poorly packed 'fluffy' layer of mitochondria was therefore discarded as much as possible during removal of the supernatant. This 'fluffy' layer might contain microsomes (cf. Schneider, 1949). The mitochondrial pellets were combined, washed with 20 ml. isotonic sucrose and resedimented at 20000 g for 10 minutes. All centrifugations were carried out using a high speed angle-head attachment for the M.S.E. refrigerator centrifuge. The temperature in the bowl

of the centrifuge was -2°. Centrifugation at 20,000 g in 0.25M sucrose does not give a very firmly packed mitochondrial sediment. It has been found convenient to obtain firmly-packed mitochondria by final sedimentation from isotonic (0.154M) KCl. The wet weight of the mitochondrial pellet was determined after decanting the supernatant fluid and carefully drying the walls of the centrifuge tube with filter paper. The mitochondria were finally thoroughly dispersed in 9 volumes 0.154M KCl to give approximately 100 mg. wet weight mitochondria per ml. Final sedimentation of the mitochondria from isotonic KCl does not appear to affect their 11β-hydroxylating activity. This sedimentation of mitochondria from isotonic KCl does, however, alter the morphological picture resulting in clumping of the mitochondria. This clumped mitochondrial preparation can be broken down on thorough stirring.

Preparations from the last sucrose wash were occasionally examined under a phase-contrast microscope. Whole cells were never observed, and single nuclei very occasionally. Purity of the mitochondrial preparations was also occasionally checked by a final centrifugation of part of the preparation from 0.01% solution of janus green B

prepared in 0.25M sucrose. On incubation for 5 minutes at 37° the blue-stained pellet in the centrifuge tube turned red. This reaction is claimed to be specific for mitochondria (Potter, Rechnagel & Hurlbert, 1951). The pellet is not disturbed in this test and if nuclei or microsomes are present as contaminants they may be distinguished at the lower and upper surfaces of the pellet respectively by their staining characteristics which differ from those of mitochondria. The mitochondrial preparations prepared as above show little trace of nuclei or microsomes and stains almost entirely red.

c) Incubation media.

All solutions used in enzymic experiments were prepared in glass-distilled water and all reagents were of AR grade.

In all experiments 25 ml. conical flasks were used as incubation vessels. Unless otherwise stated they were charged with the following reaction mixture:- 0.095M-KCl, 0.004M-$MgSO_4$, 0.04M potassium phosphate, pH 7.4, and a mitochondrial suspension in 0.154M-KCl solution containing about 1.4 mg. total nitrogen. The total volume was 3 ml. Members of the citric acid cycle, cofactors and inhibitors were added as potassium salts wherever

possible in solution at pH 7.4. When additions were made to the reaction mixture, the amount of KCl present was adjusted to maintain a total cation concentration of 0.141-0.151M.

d) Measurement of pH.

All measurements were made on a battery-operated pH meter (Type D-417 Muirhead & Co. Ltd.).

e) Addition of steroids.

In the past steroids have been added to incubation flasks dissolved in a small volume of organic solvent. Ethanol has been the chief solvent employed. However, Kochakian (1951) found that ethanol in low concentration stimulates respiration of liver and kidney slices, whereas at high concentration respiration is inhibited. Propylene glycol (propane-1:2-diol) is being used increasingly in place of ethanol (Kahnt & Wettstein, 1951; Taylor, 1954). Its high solvent power for steroids permits the addition of only very small volumes and for this reason it is regarded as a suitable solvent for in vitro work, no inhibitory effects having been reported so far. Progesterone and DOC were thus added to incubation flasks as solutions in propylene glycol with about 500 µg. steroid in 0.04 ml. The propylene glycol solution was added from a Burroughs

Wellcome 'Agla' microburette immediately after addition of the mitochondrial preparation. The best results were obtained in this way and it seems likely that the steroid is precipitated out of solution onto the mitochondrial particles and is thus more readily metabolized.

Two 0.01 ml. volumes of these solutions were pipetted into test tubes, 10 ml. ethanol were added and this solution analysed for steroid content in the spectrophotometer. The amount of steroid added to incubation flasks could thus be calculated by reference to a suitable calibration curve.

f) The general arrangement of incubation experiments.

Unless otherwise stated 'incubation', 'control' and 'blank' vessels were prepared and the following procedures adopted.

'Incubations' These particular flasks were charged with the reaction mixture desired. Mitochondrial suspension (1.0 ml.) was added to the flasks, followed by the steroid solution. When all the flasks were prepared they were closed with rubber bungs and transferred to an incubation bath at 37°. Shaking at a frequency of about 60/minute was continued for the incubation period.

'Extraction controls' Every experiment included duplicate flasks to which progesterone or DOC was added after incubation to permit a check to be made on the extraction procedure. 'Apparent' steroid levels in mitochondrial preparations were determined in incubation flasks without added steroid ('blanks'). The values for 'apparent' steroid were found to be consistently low and were mainly due to solvent residues.

g) Steroids

1) Progesterone (Organon) was recrystallized twice from n-hexane and aqueous ethanol to give a product melting at 121-121.5°.

2) DOC was prepared from 11-deoxycorticosterone acetate (Organon) by the method of Mattox & Kendall (1951). DOC was recovered from the acid hydrolysate by extraction with chloroform. The residue obtained after distillation of the washed chloroform extract was crystallized from ether and ether : acetone (Reichstein & Euw, 1938) to give a product melting at 138-142°, unchanged by admixture with an authentic sample of DOC.

$[\alpha]_D^{25}$ + 178.8±1.2° in ethanol (c. 0.493).

Steiger & Reichstein (1937) reported $[\alpha]_D^{22}$ + 178±3° for DOC in ethanol (c. 1.5).

Found : C,76.3, H,9.2

Calculated for $C_{21}H_{30}O_3$: C,76.2, H, 9.1

Portions of the DOC preparation were run on paper-strip chromatograms using solvent systems A and B_1 of Bush (1952). With both these solvent systems the DOC preparation acted as a single substance when the chromatograms were developed with either the methanolic-NaOH reagent of Bush (1952) or the blue tetrazolium reagent of Mader & Buck (1952).

3) Corticosterone (Upjohn) was a pure substance melting at 179-181°.

4) 11ß-Hydroxyprogesterone (Upjohn) was a crystalline specimen, melting point 187-189°. Reichstein & Fuchs (1940) reported melting point of 187-188° for this steroid.

h) Adenosine triphosphate (ATP).

A commercial preparation of the sodium salt (L. Light & Co. Ltd., Slough) was used. This preparation contains at least 40% adenosine diphosphate (ADP).

i) Melting points.

All melting points were determined on an

improvised hot-stage apparatus of the Kofler type (Klyne & Rankeillor, 1947). The apparatus was calibrated with pure substances of known melting point. The melting points recorded are therefore corrected.

j) Specific rotations.

These were determined for the sodium D line using a 0.5 dm. microtube. The errors are calculated as described by Klyne & Patterson (1948).

THE DETERMINATION OF PROGESTERONE, DOC, CORTICOSTERONE AND 11β-HYDROXYPROGESTERONE IN REACTION MIXTURES.

1) Introduction.

The steroids present in reaction mixtures were determined by an adaptation of the method described by Taylor (1954) for progesterone. With regard to the extraction of the steroids from reaction mixtures, the following adaptations were introduced by the author.

2) Protein precipitation.

15 ml. cold acetone were added to each incubation flask to terminate the reaction and precipitate protein. The protein in these flasks could then be filtered off immediately, whereas in Taylor's method, the flasks are allowed to stand at -20° before filtration as this results in a significant reduction in the amount of the final residue, presumably owing to the elimination of most of the phospholipid fraction. Taylor was working with amounts of liver homogenates from which a very bulky residue was obtained at the final stage if the flasks were not chilled prior to filtration. Often the partition columns used were overloaded and 'broke

down'. The amount of mitochondria to be used in the experiments to be described did not yield such a bulky residue and the time-consuming cooling of the flasks to -20° before filtration could be eliminated. The contents of the flasks were filtered with suction through Whatman No. 1 paper into 100 ml. QQ flasks using the adaptor described by Taylor (1954). The precipitate and paper were transferred to the original flask and washed with three 10 ml. volumes of acetone. Taylor had used hot acetone for this extraction, cooling the flasks to -20° before filtration. This time-consuming procedure was not necessary in these experiments using mitochondrial preparations. The combined filtrates were concentrated to aqueous residue by removal of the acetone in a stream of air at about 60-70°.

3) Extraction of aqueous residue.

Taylor (1954) used n-hexane : $CHCl_3$ (9:1, v/v) for the extraction of steroids from the aqueous residue. In the experiments to be described involving more polar steroids than progesterone, it was found that low recoveries were obtained. This was corrected by using benzene : $CHCl_3$ (6:1, v/v) as the extractent. Otherwise the method used for the extraction of steroids from the aqueous residue was

the same as used by Taylor, apart from slight differences in the volumes of solvents used.

To the aqueous residue in the 100 ml. R.B. flask were added 8 ml. of benzene -$CHCl_3$. The mixture was then transferred by suction tube to the extraction tube (cf. Taylor, 1954) and then rinsed with 3 x 5 ml. benzene -$CHCl_3$, 1 x 5 ml. water and finally with 5 ml. benzene -$CHCl_3$, each rinsing being transferred to the extraction tube. The use of benzene -$CHCl_3$ in place of n-hexane-$CHCl_3$ leads to an increased tendency to form emulsions. These emulsions, however, were rapidly cleared by centrifugation. Following centrifugation the upper phase was transferred by means of another suction tube (Taylor, 1954) to the original 100 ml. flask and the aqueous phase extracted with 2 x 20 ml. benzene-$CHCl_3$. The combined extracts were evaporated to dryness in an air stream at about 90°, removal of the last traces of water being facilitated by addition of ethanol.

The above procedure although following the method developed by Taylor does differ slightly in the volumes of solvent used and does not involve hot extraction. In this way the extraction procedure is much quicker, an important factor in experiments involving many steroid determinations.

4) Application of extract to partition columns.

After addition of 5 ml. 'mobile phase' the flasks were left overnight at 20° in the temperature-controlled room used for the partition chromatography. It was found convenient to leave the extract overnight in contact with the 'mobile phase' to ensure complete solution. Taylor (1953) found that a minimum of 6 hours was required to dissolve his extracts completely. More recently it has been found that in the estimation of DOC and corticosterone the 'mobile phase' will dissolve the extract completely in about 1 hour, and thus partition chromatography can be done on the extracts the same day as the extraction procedure, although in most cases it is convenient to leave the flasks overnight in contact with 'mobile phase'.
To each partition column was added 0.5 ml. of the extract solution. This was allowed to disappear just below the surface of the celite, then the column was washed down with 2 ml. 'mobile phase'. After this rinsing had entered the celite about 5 ml. 'mobile phase' was added to the column and a reservoir containing 'mobile phase' was fitted to the column. The eluate was collected from the addition of the extract and formed part of the first fraction. The rates of flow of the columns used fell within the range 15-25 ml./hr. (The rate of flow was determined

after addition of the extract since the addition of extract markedly decreases the flow rate.)

In the partition chromatographic method developed by Taylor for the determination of progesterone, the first 10 ml. eluate were rejected and the next 12 ml. collected for progesterone analysis. In developing partition chromatographic systems for the estimation of DOC, corticosterone and 11β-hydroxyprogesterone the principle of Taylor's method has been followed in that, in general, the first 10 ml. eluate contained non-polar lipid and other interfering material. The next 15-20 ml. was then collected and this contained the steroid in question.

The steroid fractions were taken to dryness in an air stream at about 80° and the tubes dried <u>in vacuo</u> over $CaCl_2$ for at least 30 min. (This final drying is to remove last traces of benzene which interferes with the spectrophotometric estimation of the steroids.) The residues were dissolved in 5 ml. ethanol and the optical density of the ethanol solutions were read at 240 mμ against pure ethanol in the compensating cell. 1 cm. cells of a Unicam S.P. 500 Spectrophotometer were used. The amounts of steroids present were found by reference to calibration curves prepared by measuring the optical densities of solutions of known steroid concentration.

5) Column partition chromatography.

a) Purification of celite.

Celite 545 (Johns Manville & Co.) was purified as described by Taylor (1953).

b) Preparation of columns.

The method used for packing columns was that described by Butt, Morris, Morris & Williams (1951) and Taylor (1953). As described by Taylor, the supporting phase (celite) and the appropriate amount of stationary phase are mixed and left overnight in a stoppered jar in the temperature controlled room. This is to ensure even distribution of the stationary phase. Morris (1953) has shown that this is unnecessary and that when the mobile phase is subsequently added to this mixture to form a slurry, the stationary phase becomes evenly distributed throughout the supporting phase. In order to obtain a uniformly packed column it was necessary to rotate the column about 45° after every pack. This is necessary as the columns vary in width. If the column is not rotated during the packing it is packed more firmly on one side than the other, resulting in uneven flow of solvent and material through the column. One component of the extracts chromatographed remains at the top of the column during development of the

chromatogram and, if the column is loosely packed at one side, this material streaks down the column. If the column is evenly packed by rotating the column during the packing operation, this material is seen as a narrow even band around the top of the column. The elution patterns of steroids from unevenly packed columns are irregular, tending to show band spreading.

c) Separation of DOC from corticosterone and adrenocortical extract.

The chromatographic technique used was an adaptation of that described by Taylor (1954). Preliminary experiments were carried out to find a solvent system which could be used to separate DOC and corticosterone on a column 10 cm. long and 1.0 cm. diameter. Approximately 5 g. celite is needed to pack such a column, and 5 ml. stationary phase is used per 5 g. celite. The solvent systems tried were based on those described by Butt, Morris, Morris & Williams (1951) having an aqueous-methanol stationary phase. A stationary phase composition of methanol-water (7:3, v/v) was selected as a constant and the composition of the mobile phase varied to give a partition coefficient for DOC of about 2-2.5. It was found by Taylor (1953) that with such partition columns a steroid having a partition coefficient of about 2 in the system employed would

have a retention volume of about 15 ml. i.e. the volume of eluate to peak concentration of steroid would be about 15 ml. Lipid material in the extracts containing the steroids was eluted in the first 6 ml. allowing one to discard the first 10 ml. eluate and collect the next fraction which would contain the steroid in question with a partition coefficient about 2 in the particular solvent system employed. In the case of progesterone isolation on a partition column using n-hexane as mobile phase and methanol:water (7.5:2.5, v/v) as stationary phase, the first 10 ml. eluate are rejected and the next 12 ml. collected for progesterone analysis.

In the separation of DOC from adrenocortical extract the polarity of the mobile phase has to be increased as compared with progesterone, in order to obtain a retention volume of 15 ml. under these conditions. However, DOC has also to be separated from corticosterone present in the same extract. In the system with benzene:n-hexane (4:6, v/v) as mobile phase and methanol:water (7:3, v/v) as stationary phase DOC has a partition coefficient of 1.9 and corticosterone 35 for the stationary phase. It seemed possible to use this system to separate DOC from lipid material and corticosterone. When 220 µg. each of DOC and corticosterone added in 0.5 ml. mobile phase were chromatographed using this solvent

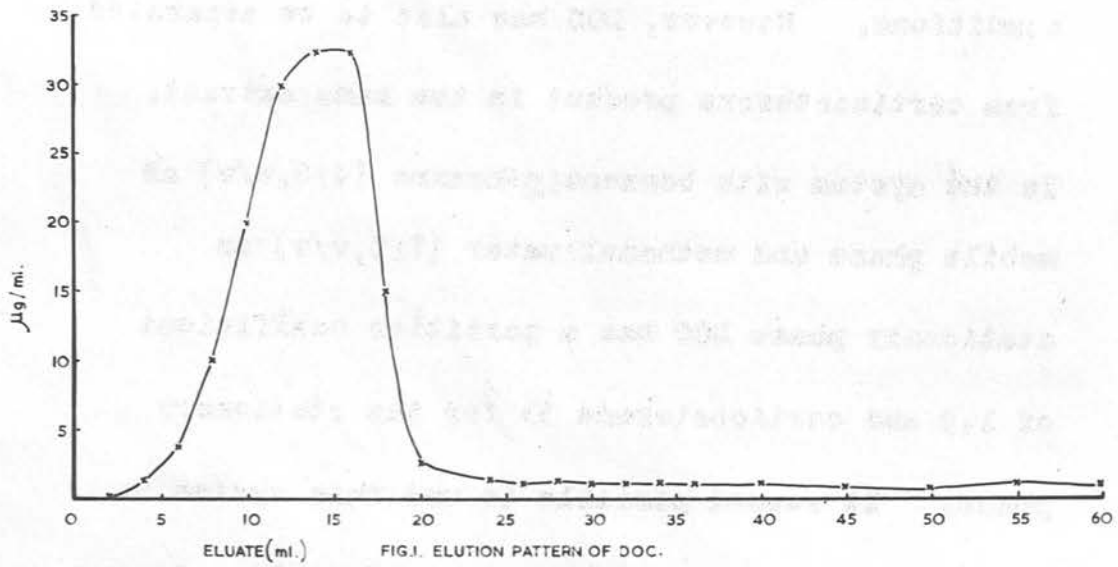


FIG.1. ELUTION PATTERN OF DOC.

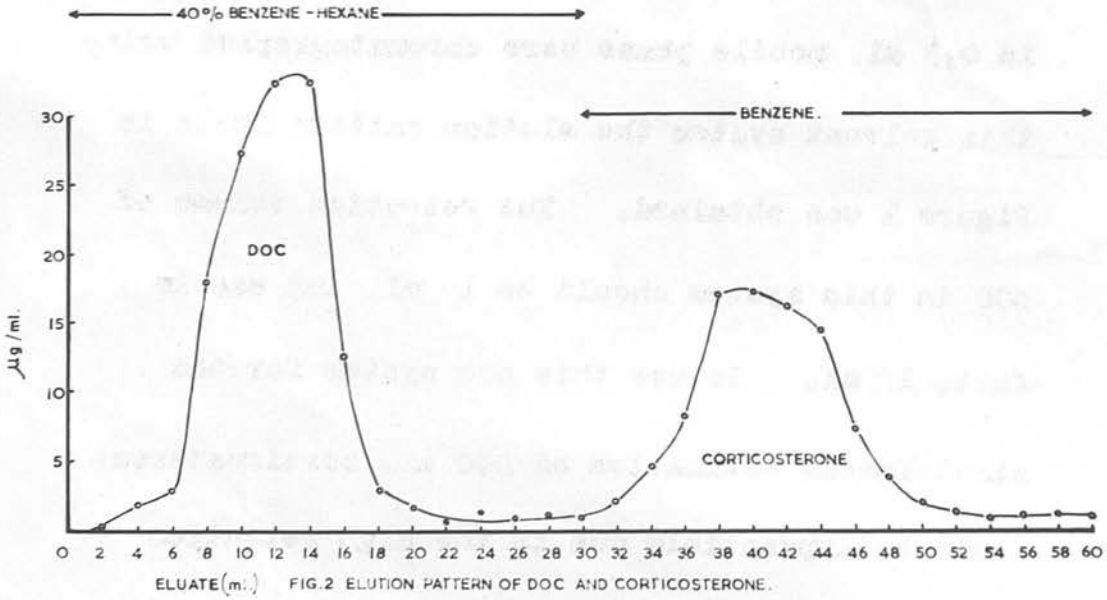


FIG.2 ELUTION PATTERN OF DOC AND CORTICOSTERONE.

system the elution pattern shown in Fig. 1 was obtained. The retention volume of DOC in this system should be 10 ml. and was in fact 15 ml. To use this one system for the simultaneous estimation of DOC and corticosterone would be impractible due to the high retention volume of corticosterone. The composition of the mobile phase was therefore changed after the elution of DOC to give a mobile phase of benzene. The benzene was previously equilibrated with stationary phase to prevent, as much as possible, alteration of stationary phase composition on changing to a mobile phase of benzene. As seen in Fig. 2 a very efficient separation of DOC and corticosterone is obtained by this procedure. Although having a retention volume of 10 ml. in this system DOC elution starts after 4 ml. In extracts of adrenocortical mitochondria, non-polar lipid material is also eluted at this point and would interfere with the DOC estimation. The system with benzene:n-hexane (3.5:6.5, v/v) as mobile phase and methanol:water (7:3, v/v) as stationary phase was tried. With this system the DOC elution starts after 10 ml., thus leaving a safer margin between DOC and non-polar lipid material which itself was eluted from 4-8 ml. However, it was decided to reduce the benzene concentration of the mobile phase still further to

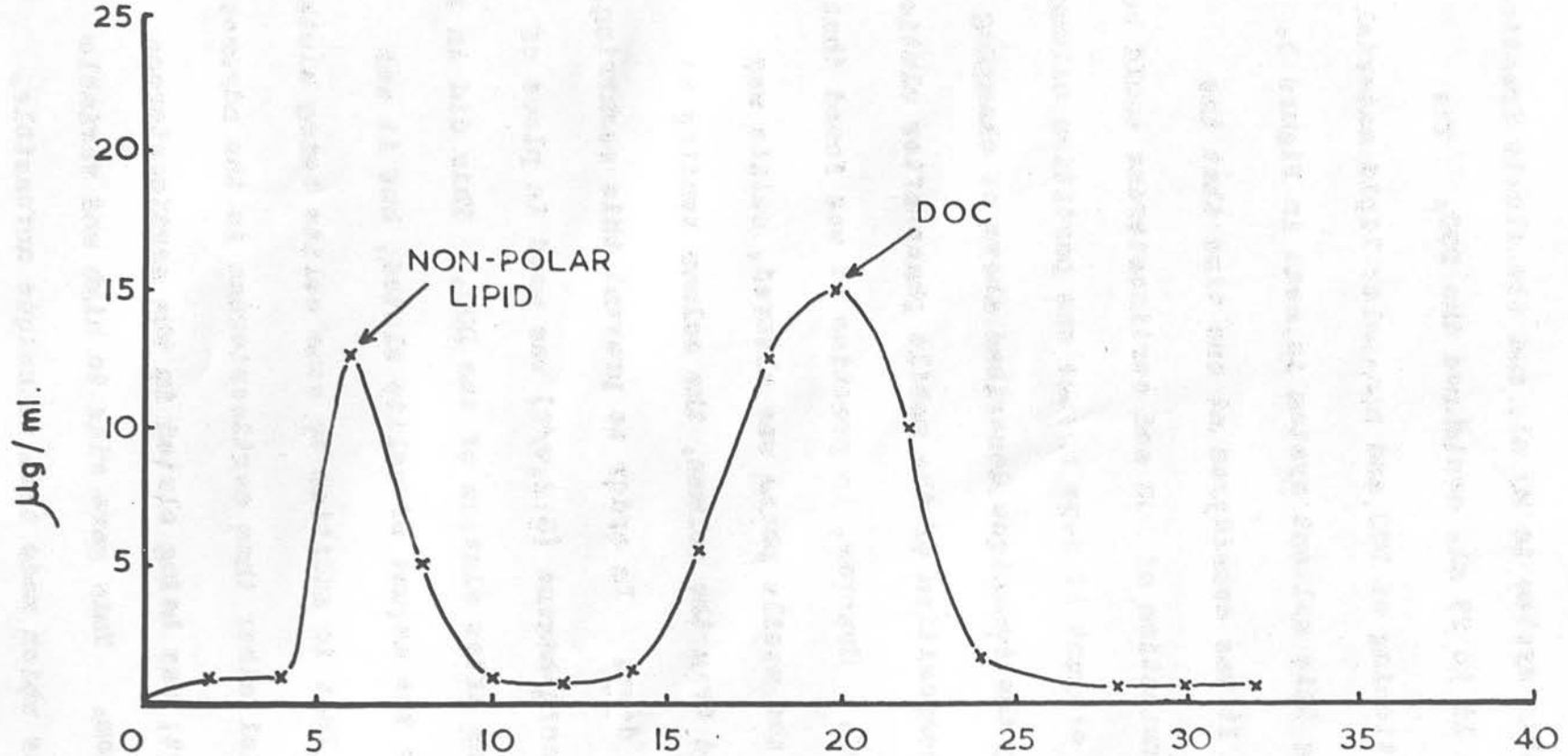


FIG.3. SEPARATION OF DOC FROM NON-POLAR LIPID.

allow rejection of the first 10 ml. eluate without this cut containing even traces of DOC. The system finally used for the separation of DOC from extracts has benzene:n-hexane (3:7, v/v) as mobile phase, and methanol:water (7:3, v/v) as stationary phase. The retention volume of DOC in this system is 20 ml. and the eluate fraction from 15 to 25 ml. contained the DOC. The positioning of DOC and non-polar lipid material using this solvent system is seen in Fig. 3.

It was considered at one time that the determination of DOC and corticosterone would be more elegant if done on the one partition column, using the technique described above of changing the composition of the mobile phase after elution of DOC. However, in practice it was found that when the mobile phase was altered, celite was eluted from the column, the column tending to break down. In order to prevent this occurring, benzene:n-hexane (8:2, v/v) was used in place of benzene after elution of the DOC. This did in fact reduce the amount of celite eluted, but it was found that in addition to some celite being eluted, material other than corticosterone in the adrenocortical extract was being eluted in the corticosterone fraction. This gave rise to high and variable blanks which made this technique unfeasible. Corticosterone was therefore determined on a separate

SEPARATION OF CORTICOSTERONE AND DOC.

MOBILE PHASE :- BENZENE.
STATIONARY PHASE :- METHANOL - WATER (7:3)

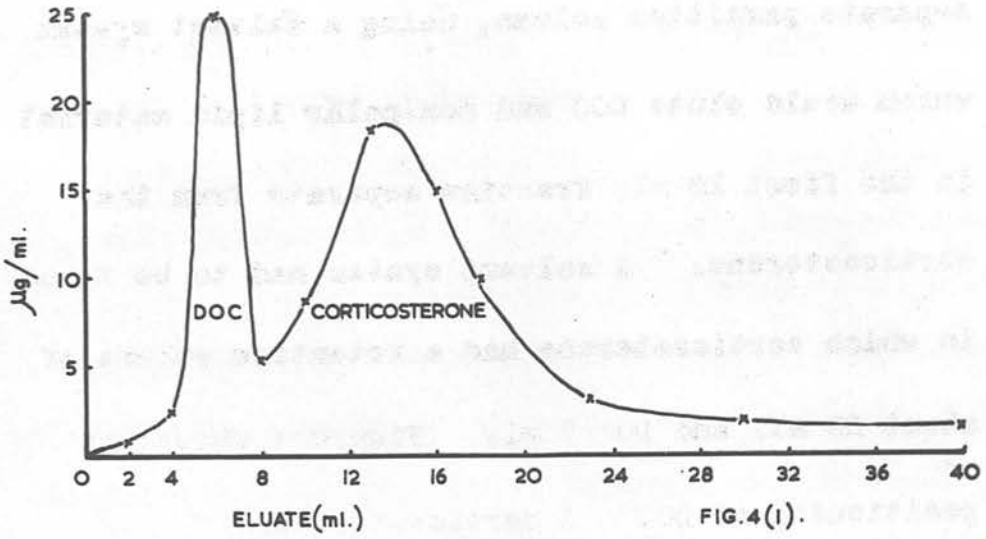


FIG. 4 (1).

MOBILE PHASE :- BENZENE - HEXANE (9:1)
STATIONARY PHASE :- METHANOL - WATER (7:3)

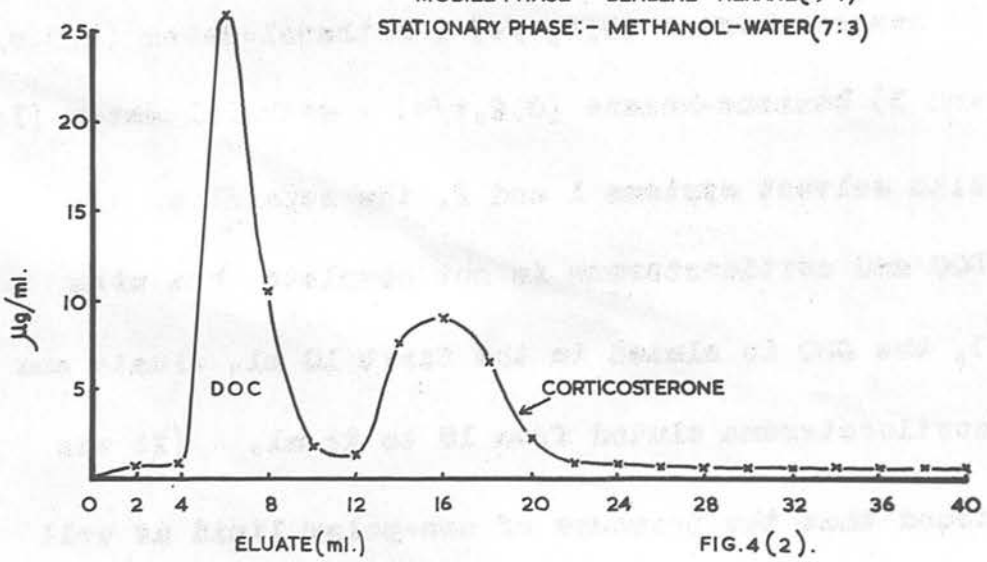


FIG. 4 (2).

MOBILE PHASE :- BENZENE - HEXANE (8:2)
STATIONARY PHASE :- METHANOL - WATER (7:3)

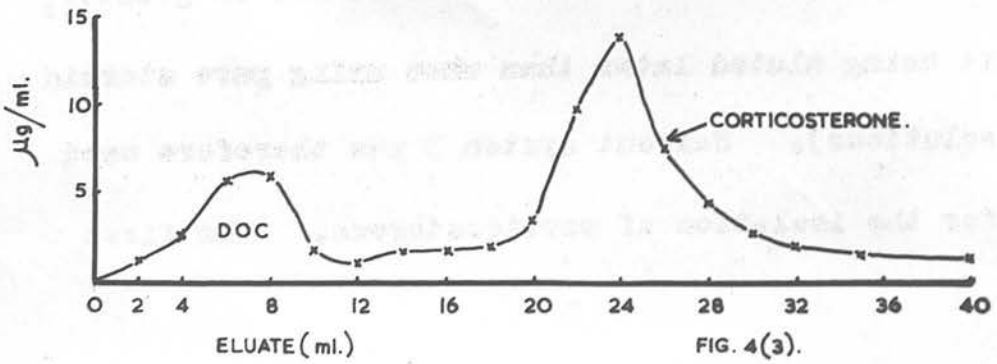


FIG. 4 (3).

partition column, using a solvent system which would elute DOC and non-polar lipid material in the first 10 ml. fraction separate from the corticosterone. A solvent system had to be found in which corticosterone had a retention volume of about 20 ml. and DOC 5 ml. Fig. 4 shows the positioning of DOC and corticosterone with the solvent systems

1) benzene:methanol-water (7:3, v/v);

2) benzene-hexane (9:1, v/v) : methanol-water (7:3,v/v);

3) benzene-hexane (8:2, v/v) : methanol-water (7:3,v/v).

With solvent systems 1 and 2 the separation of DOC and corticosterone is not complete, but with 3 the DOC is eluted in the first 10 ml. eluate and corticosterone eluted from 18 to 26 ml. (It was found that the presence of non-polar lipid as well as steroids markedly affected the elution pattern of these steroids. In the presence of lipid material the retention volume of DOC is greater, it being eluted later than when using pure steroid solutions.) Solvent system 3 was therefore used for the isolation of corticosterone. The first 12 ml. eluate were rejected and the next 20 ml. (corticosterone fraction) were collected for analysis.

d) Separation of progesterone and 11β-hydroxy-progesterone on partition columns.

Progesterone in reaction mixtures was determined by the method described by Taylor (1954). 11β-Hydroxyprogesterone present in reaction mixtures along with progesterone was determined using a separate partition column and a different partition system. The partition system used was one in which progesterone was eluted in the first fraction along with non-polar lipid material. This fraction was discarded. The solvent system used was the DOC system having benzene:n-hexane (3:7, v/v) as mobile phase and methanol:water (7:3, v/v) as stationary phase. The partition coefficient of 11β-hydroxy-progesterone in this system is 4, whereas DOC has a partition coefficient of 2.6. The retention volume of 11β-hydroxyprogesterone using this system is 40 ml. which would involve a somewhat lengthy chromatogram run. The amount of stationary phase used was therefore reduced to 4 ml. per 5 g. celite. In this way the 11β-hydroxyprogesterone was eluted sooner having a retention volume of 32 ml. The 11β-hydro-progesterone was eluted between 26-35 ml. The first 20 ml. eluate containing progesterone and non-polar lipid material was discarded and the next 20 ml.

collected for analysis. This was the 11β-hydroxyprogesterone fraction.

6) Accuracy of the methods.

Taylor (1953) checked the accuracy of the method in a series of experiments in which known amounts of progesterone were recovered from liver slices and homogenates. In the present author's experience the recovery of progesterone from ox-adrenocortical mitochondria was consistently high and, at the 500 μg. level, steroid recoveries of 94-98% were obtained. These recoveries are similar to those obtained by Taylor.

The accuracy of the methods for DOC, corticosterone and 11β-hydroxyprogesterone was checked by recovery of known amounts of these steroids from ox-adrenocortical mitochondrial preparations. Varying amounts of these substances were added to flasks containing 100 mg. wet weight mitochondria and incubation medium. Chilled acetone was immediately added and the flasks worked up by the methods already described.

RESULTS

a) Recovery of DOC.

DOC added (µg.)	DOC recovered (µg.)	(%)	(corrected for 'blank')
524	500	96	
	506	97	
540	526	97	
	511	94	
	517	95	
536	517	96.5	
	514	96	
520	500	96	
	497	96	
470	465	99	
	462	98	
456	450	98.5	
460	425	92	
	425	92	
	450	98	
138	138	100	
	138.5	100.6	
36	34	94	
	34.5	96	

b) Recovery of corticosterone.

Corticosterone added (µg.)	Corticosterone recovered (µg.)	(%)	(corrected for 'blank')
125	122	97.6	
	121.5	97.2	
99	97	98	
	98	99	
36	33	92	
	33	92	
	32.5	91	

c) Recovery of 11β-hydroxyprogesterone.

11β-Hydroxyprogesterone added (µg.)	11β-Hydroxyprogesterone recovered (µg.)	(%)	(corrected for 'blank')
406	386	95	
	389	96	
235	210	89	
	205	87	

The results obtained show that the methods employed are satisfactory for the determination of these steroids at the 100 µg. level upwards. Below the 100 µg. level of steroid the spectrophotometer readings are low enough to be considerably affected by any slight variation in the 'blank'. In order to get higher spectrophotometer readings more extract has to be chromatographed and this frequently overloads the column and causes breakdown.

7) Specificity of the method.

Taylor (1953) has discussed the specificity of the method as applied to progesterone and similar arguments can be applied for DOC, corticosterone and 11β-hydroxyprogesterone. The chief factors determining the specificity of the methods are:-

1) The partition coefficients of these steroids in the solvent systems used.

2) The selective absorption at 240 mμ. of the α:β-unsaturated ketonic grouping in the steroid A ring.

In the estimation of DOC this steroid has to be separated from corticosterone and also relatively non-polar lipid material. As seen above this is easily effected. In the estimation of corticosterone this compound is readily separated from DOC and non-polar lipid. However, the chromatographic procedures used in the determination of these substances are not as critical as would have to be used if steroids such as 6β-hydroxy-11-deoxycorticosterone, 17α-hydroxy-11-deoxycorticosterone and 11-dehydrocorticosterone were also produced on incubation of DOC with ox-adrenocortical mitochondria. However, it has been shown that incubation of DOC with ox-adrenocortical mitochondria apparently results in

its conversion to corticosterone alone. The enzymes catalysing 17 and 21-hydroxylation of steroids in the adrenal gland are situated in the 'supernatant fraction' of a homogenate centrifuged at 20000 g (Plager & Samuels, 1953). Under the incubation conditions used, no 6β-hydroxylation of DOC has been found using these mitochondrial preparations. No evidence has been found for the production of 11-dehydrocorticosterone on incubation of DOC with ox-adrenocortical mitochondria, but this substance has been obtained when DOC was incubated with hog adrenal breis (Hechter et al., 1951). It appears that by using mitochondrial preparations one can restrict hydroxylation to the 11 position in the steroid nucleus. The fact that the sole metabolic product of DOC under these conditions is corticosterone, means that one has the very simple separation to effect of DOC from corticosterone, a relatively much more polar steroid. In the solvent partition systems used for the determination of DOC and corticosterone, these substances have vastly different partition coefficients which ensures their separation. With such an easy separation the chromatographic technique used is rapid though efficient. This speed of operation is very important in experiments involving up to 24 steroid estimations.

In the estimation of 11β-hydroxyprogesterone one again has a relatively simple system in that 11β-hydroxyprogesterone is the main metabolite of progesterone incubation with ox-adrenocortical mitochondria. Substances which would be most likely to interfere with the estimation of 11β-hydroxy-progesterone are DOC, 17α-hydroxyprogesterone and 11α-hydroxyprogesterone. As will be shown later no DOC or 17α-hydroxyprogesterone is produced on incubation of progesterone with these mitochondrial preparations. The mitochondria do not contain the enzyme systems required for the insertion of hydroxyl groups in the 17α- and 21- positions. Traces of more polar steroid material have been found on incubation of progesterone with ox-adrenocortical mitochondria, but this cannot interfere with the estimation of 11β-hydroxyprogesterone with the partition system used. As seen above, 11β-hydroxyprogesterone is very readily separated from progesterone and non-polar lipid material, due to the great difference in partition coefficients of these substances in the solvent partition system used for 11β-hydroxyprogesterone isolation. In the same way, 11β-hydroxyprogesterone does not interfere with the separation of progesterone from incubation mixtures. Reduction of progesterone in the A ring,

and/or at the 20 position would give products which would be included in the progesterone fraction following chromatography, but none of these compounds absorb significantly at 240 mμ and so would not interfere with progesterone estimation. In addition, most evidence points to such reductions being catalyzed by enzymes in the supernatant fraction of a fractionated homogenate of liver (Atherden, 1954), but not of adrenal.

8) The nature of the blank.

In all incubation experiments 'blank' flasks were included containing the enzyme preparation and incubation medium but no steroid. These flasks were worked up by the usual procedure, and on chromatography a fraction corresponding to the steroid fraction was collected. The optical density of this fraction, when taken to dryness and dissolved in ethanol, was substracted in all steroid determinations. This 'apparent' steroid in these 'blank' incubations varied between 10-30 μg. This blank was chiefly due to solvent residues arising, in particular, from the benzene. In practice it was possible to purify the solvents used sufficiently to keep the 'blank' consistently low. The 'blank'

fractions after chromatography do not show selective absorption at 240 mμ and their absorption spectrum over the range 225-300 mμ is a smooth curve which decreases with increasing wavelength.

9) Paper chromatography.

Solvent systems of the type used by Bush (1952) were employed. Bush advocates the running of paper chromatograms at 37° to obtain quick runs with good resolution of mixtures. At first chromatograms were run at this elevated temperature making use of the 'chromatocoil' of Schwarz (1952), in which paper strips of about 1 cm. width are placed in spiral form in containers which were readily accommodated in an incubator at 37°. Two paper strips could be run in each 'chromatocoil' jar, thus 'standard' and 'unknown' steroids could be run under identical conditions.

More recently, paper chromatograms have been run in glass tanks, similar to those used by Bush (1952). The chromatograms were run at about 20° in a temperature controlled room. Sheets of Whatman No. 1 or No. 42 (40 cm. long, 15 cm. broad) were used and the general chromatographic technique was that used by Bush (1952).

The reagents for the detection of the steroids were:-

a) Methanolic-NaOH reagent of Bush (1952) which detects steroids having an αβ-unsaturated ketonic grouping in the A ring in a concentration of 2 μg./cm².

b) Blue tetrazolium reagent (Mader & Buck, 1952) which detects steroids having an α-ketol side chain in a concentration of 1-2 μg./cm².

c) 15% phosphoric acid (Neher & Wettstein, 1951) which detects steroids having an αβ-unsaturated ketonic grouping in the A ring in concentrations of 5-20 μg./cm² depending on the steroid.

10) Ultraviolet spectroscopy.

The technique used was the standard one described by Taylor (1953) for the estimation of steroids following chromatography. The instrument used was the Unicam Photoelectric Quartz Spectrophotometer, S.P. 500 (Unicam Instruments, Cambridge, Ltd.). Using this instrument, advantage was taken of the selective absorption at 240 mμ of the steroids used.

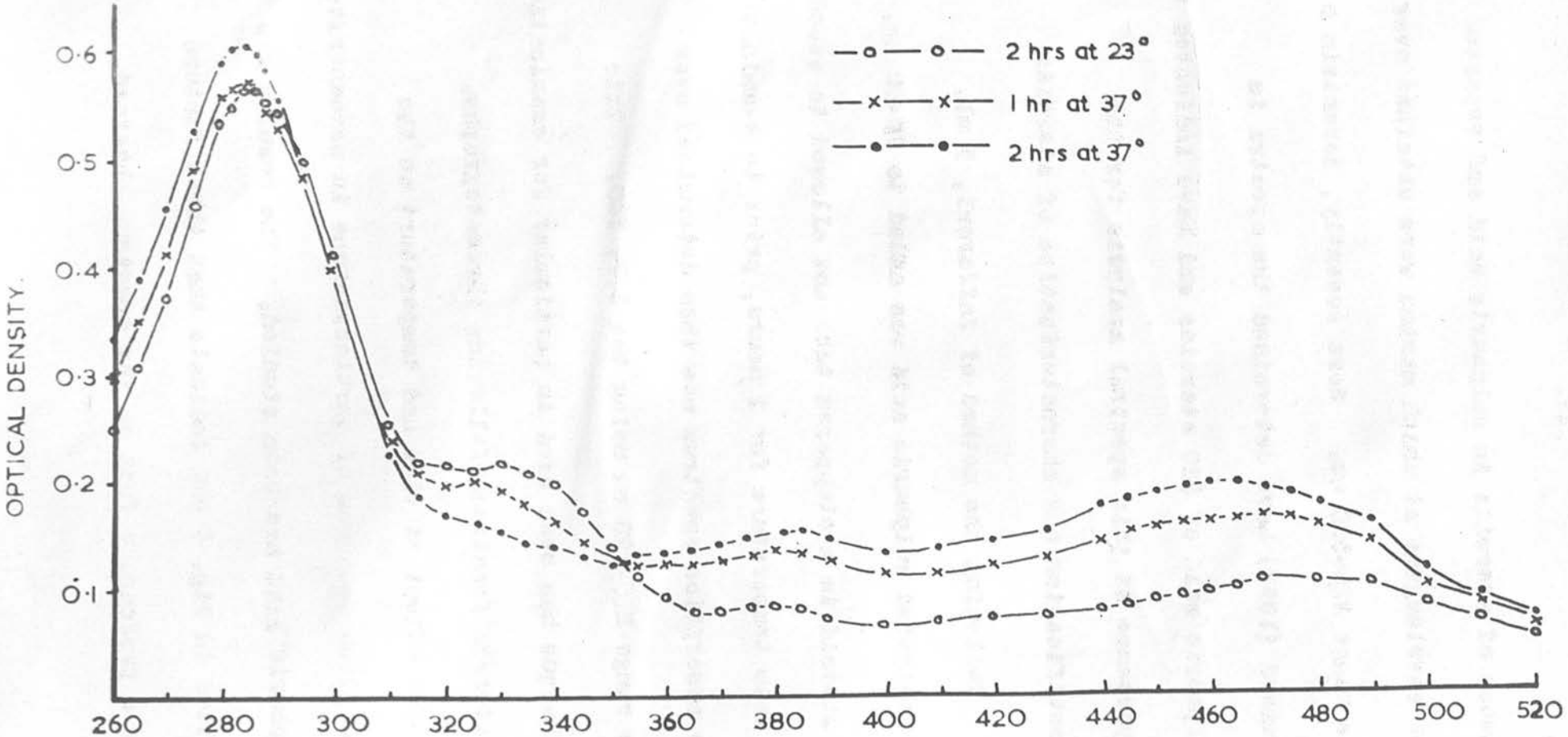


FIG. 5. EFFECT OF TIME AND TEMPERATURE ON H_2SO_4 CHROMOGEN OF CORTICOSTERONE.

11) The absorption spectra of steroids in concentrated H_2SO_4.

Zaffaroni (1950) determined the spectra of a number of steroids in sulphuric acid and reported the wavelengths at which maxima were obtained over the range 220-500 mμ. More recently, Bernstein & Leuhard (1953) have determined the spectra in sulphuric acid of 220 steroids and have indicated the importance of this spectral analysis for the identification and characterization of steroids.

Following the method of Zaffaroni, 5 ml. concentrated sulphuric acid was added to 70-90 μg. of steroid in a stoppered tube and allowed to stand at room temperature for 2 hours prior to examination. The absorption spectrum was then determined over the range 220-520 mμ using the S.P. 500. This technique has been used in particular for examination of steroid fractions following chromatography.

The effect of time and temperature on the absorption spectrum of corticosterone in concentrated sulphuric acid has been studied. The results are plotted in Fig. 5, and indicate that these factors have a profound effect on the spectrum obtained. The absorption maxima obtained with corticosterone at 330 and 460 mμ are particularly affected. Incubation for 2 hours at 37°, which can be used

successfully with steroids like 11β-hydroxyprogesterone, leads to flattening of the maxima at 330 and 460 mμ. The effect of time and temperature is to alter the general shape of the spectrum obtained with corticosterone. In general, the conditions used for development of the sulphuric acid chromogens have been 2 hours standing at room temperature.

SECTION I

THE METABOLISM OF 11-DEOXYCORTICOSTERONE BY OX-ADRENOCORTICAL MITOCHONDRIA.

1) Identification of corticosterone as the product obtained on incubation of DOC with ox-adrenocortical mitochondria.

In the experiments to be described in this section DOC was incubated with adrenocortical mitochondria in reaction mixtures containing 2 mM-fumarate and the usual reaction mixture previously described. Following the incubation of DOC under these conditions the usual extraction procedure was used, and DOC and corticosterone determinations done on the final extract. The metabolic product eluted from Celite partition columns in the 'corticosterone fraction' absorbed selectively at 240 mμ and accounted quantitatively for the amount of DOC which had disappeared.

RESULTS

DOC recovered		Corticosterone recovered		Total recovery	
(µg.)	(%)	(µg.)	(%)	(µg.)	(%)
After incubation					
90	15.8	448	78.7	538	94.5
96.5	17	455	80	551.5	97
90	15.8	452	79.3	542	95
80	14	460	80.7	540	95
Extraction control					
538	94.4				
DOC added					
570					

In support of this suggestive evidence that the single metabolic product is corticosterone the material eluted in the 'corticosterone fraction' was run on paper-strip chromatograms using solvent systems B_1, B_4 and B_5 of Bush (1952). The presence of steroid on the paper chromatograms was shown by development with the 15% phosphoric acid reagent. With this reagent

authentic corticosterone gives a green fluorescence when viewed in ultra-violet light. Mixed chromatograms of authentic corticosterone and metabolic product, metabolic product alone, and authentic corticosterone alone were run. All chromatograms showed spots with green fluorescence in ultra-violet light. These spots had all run to the same position on the paper chromatograms. These investigations were carried out using the 'chromatocoil' of Schwarz (1952). Paper chromatograms were also run in the Bush (1952) type of chromatogram tank, using descending paper chromatography. In these experiments the papers were developed with the blue tetrazolium reagent. Similar results were obtained in these investigations indicating that apparently the sole metabolic product was corticosterone. These paper chromatogram runs were done on the 'corticosterone fraction' obtained after column partition chromatography, but paper-strip chromatograms have also been run with material from the final extracts from incubation flasks. These investigations again suggested that DOC was being metabolized to a single substance which behaved similarly to corticosterone on paper chromatograms with various partition systems.

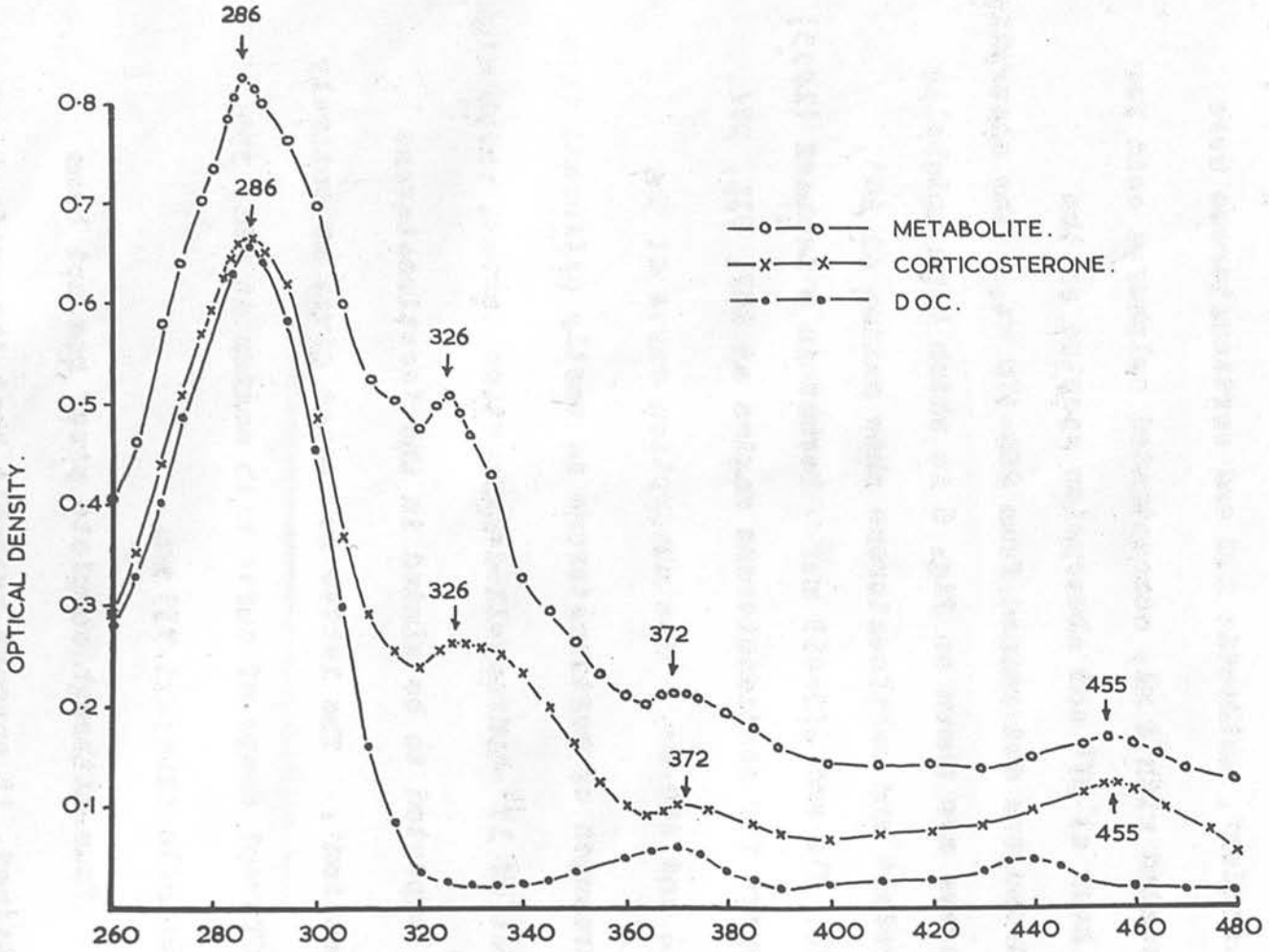


FIG.6. H_2SO_4 CHROMOGEN OF METABOLIC PRODUCT, DOC AND CORTICOSTERONE.

50 µg. portions of metabolic product isolated from Celite partition columns in the 'corticosterone fraction', authentic DOC and corticosterone were treated with 4 ml. concentrated sulphuric acid for 1 hour at 37°, and absorption spectra of the chromogens determined from 260-470 mµ. The absorption curves are shown in Fig. 6 in which both metabolic product and corticosterone show maxima at 285, 326, 372 and 455-465 mµ. Bernstein & Leuhard (1953) report for corticosterone maxima at 287, 331, 374, 418 and 460 mµ. The absorption curve of the chromogen of corticosterone is different from that of 17α-hydroxy-11-deoxycorticosterone, which might be expected to be eluted in the 'corticosterone fraction'. The latter substance gives an entirely different shape of curve with maxima at 288, 338, 486 (inflection) and 535 mµ.

Thus, although complete proof has not been obtained, it appears probable that the predominant or single metabolic product obtained in these experiments is corticosterone. In addition, Sweat (1952) has isolated corticosterone following the incubation of DOC with ox-adrenocortical mitochondria. DOC and corticosterone determinations were done in all metabolic experiments and occasionally the 'corticosterone fractions' were treated with

concentrated sulphuric acid and the absorption spectra examined. In all cases the absorption spectra corresponded with that of corticosterone.

2) Citric acid cycle intermediates as activators of DOC 11β-hydroxylation.

Several workers (Kahnt & Wettstein, 1951; Hayano, Dorfman & Yamada, 1951) have reported the activation of 11β-hydroxylation in the adrenal by certain members of the citric acid cycle. In particular, the importance of fumarate has been stressed (Hayano & Dorfman, 1953).

In the experiments now reported the effect of several citric acid cycle intermediates on 11β-hydroxylation of DOC was investigated. The effect of ATP on the 11β-hydroxylation was also examined.

RESULTS

100 mg. wet weight mitochondria (about 1.4 mg. total nitrogen): usual reaction mixture with citric acid cycle acids present as 0.002M potassium salts (pH 7.4). Incubation for 1 hour at 37°. ATP concentration was 0.5 mM.

	DOC hydroxylated	
	(µg.)	(%)
After incubation		
No activator	0	0
No activator + ATP	0	0
Pyruvate	16	3
Citrate	119	24
Oxaloacetate	202	40
Malate	300	60
Fumarate	418	84
Succinate	420	84
α-Oxoglutarate	420	84
Succinate + ATP	498	100
Extraction control	495	97
DOC added	500	

Conclusion

11β-Hydroxylation of DOC by ox-adrenocortical mitochondria requires the presence of intermediates of the citric acid cycle. Pyruvate has a negligible effect, whereas all other citric acid cycle acids tried are effective. Malate, succinate, fumarate and α-oxoglutarate are the most effective. Addition of ATP or ADP alone are without effect, but these substances enhance the 11β-hydroxylation obtained in the presence of added citric cycle intermediates. This experiment has been repeated on many occasions and the effect of ATP or ADP is to yield 100% DOC hydroxylation in the presence of succinate, fumarate and α-oxoglutarate. On certain rare occasions metabolism of DOC in the presence of fumarate was much greater than in the presence of other 'cycle intermediates'. This is not a typical result and there is evidence that this is obtained with mitochondria prepared from old adrenal glands.

The observed requirement for citric acid cycle intermediates for the DOC 11β-hydroxylation reaction suggested the possibility of a relationship between steroid 11β-hydroxylation and oxidation of the added 'cycle intermediates'. This was investigated by oxygen-consumption measurements made by the usual Warburg technique (Umbreit, 1949).

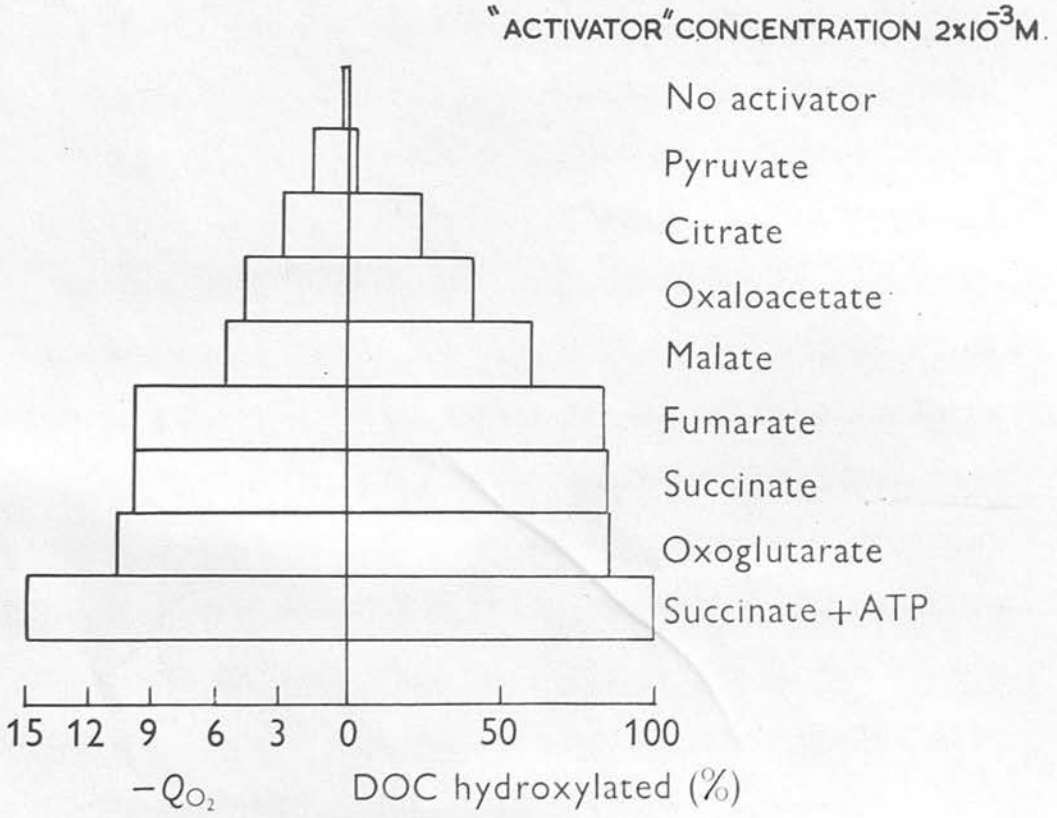

"ACTIVATOR" CONCENTRATION 2x10⁻³M.
No activator
Pyruvate
Citrate
Oxaloacetate
Malate
Fumarate
Succinate
Oxoglutarate
Succinate + ATP
15 12 9 6 3 0 50 100
$-Q_{O_2}$
DOC hydroxylated (%)

FIG. 7.

These oxygen-consumption measurements were made using the same mitochondrial preparation as was used above for the investigation of the effect of these 'cycle intermediates' on DOC 11β-hydroxylation. Identical incubation conditions were used. From the results shown in Fig. 7 it may be seen that the 'cycle intermediates' which are oxidized most rapidly are the most effective activators of DOC 11β-hydroxylation. ATP which increases the rate of oxidation of succinate, α-oxoglutarate, malate and fumarate has a corresponding stimulating effect on their ability to activate DOC 11β-hydroxylations. This is shown in Fig. 7 in the case of succinate where added ATP increases succinate oxidation, accompanied by increased DOC 11β-hydroxylation.

This experiment relating oxygen-consumption and activation of DOC 11β-hydroxylation would suggest that the activation of DOC 11β-hydroxylation in the presence of any one 'cycle intermediate' depends on the ability of the mitochondria to oxidize this intermediate.

3) Influence of DOC on oxidation of citric acid cycle intermediates in relation to DOC 11β-hydroxylation.

The interpretation of the above oxygen-consumption experiments is complicated by the observation that the oxidation of cycle intermediates is affected by the DOC present. With some citric acid cycle acids this results in marked inhibition of oxidation. The effect of this inhibition on DOC 11β-hydroxylation is seen in the experiment reported here.

RESULTS

100 mg. wet weight mitochondria (about 1.4 mg. total nitrogen). Standard reaction mixture plus 0.5 mM ATP.

	DOC added (µg.)	DOC recovered (µg.)	(%)	Q_{O_2}
After incubation				
With 2 mM-malate	0	0	-	10.6
	500	5	1	7.8
With 2 mM-oxaloacetate	0	-	-	5.6
	490	460	94	0.6

Conclusion

The use of oxaloacetate as 'activator' normally permits about 40% hydroxylation (See Fig. 7). The circumstances giving rise to the somewhat different results of the above experiment (p. 52) are not at present clear, although they may be related to variations in the age of adrenal glands used. The suppression of oxygen-consumption in the presence of DOC has resulted in failure to 11β-hydroxylate DOC. This was the first suggestive evidence that DOC itself determines to a certain extent the degree to which it is metabolized. In this experiment DOC has inhibited oxidation of oxaloacetate considerably with a marked lowering of DOC 11β-hydroxylation. With malate as 'activator' oxidation was not inhibited as markedly by the added DOC, and 11β-hydroxylation of DOC was almost complete.

4) The effect of magnesium ions on 11β-hydroxylation of DOC.

Cohen (1953) observed an increased requirement for magnesium ions when citrate oxidation is used as an energy source for certain synthetic reactions in liver homogenates. As this offered an explanation of the poor activation of DOC 11β-hydroxylation in the presence of added citrate, the effect of varying

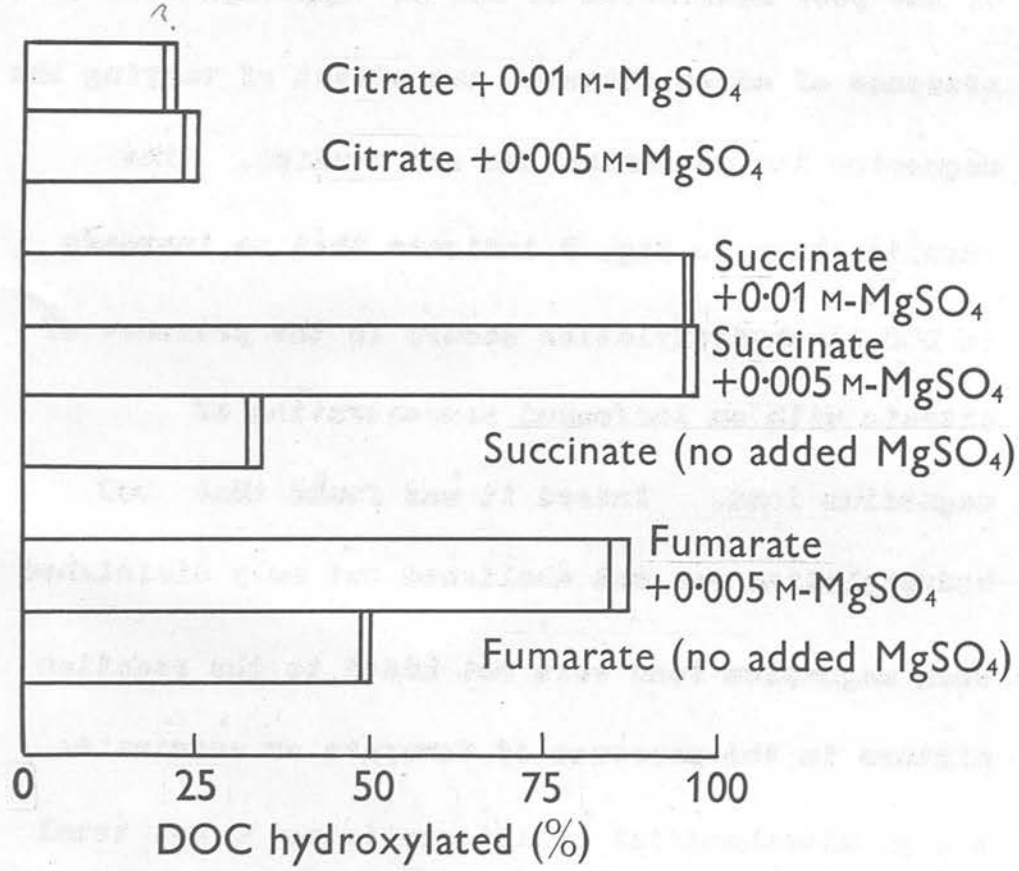


Fig.8 Efect of magnesium ions on 11β-hydroxylation

the magnesium ion concentration was studied. The results shown in Fig. 8 indicate that no increase in DOC 11β-hydroxylation occurs in the presence of citrate with an increased concentration of magnesium ions. Indeed it was found that DOC hydroxylation was not abolished but only diminished when magnesium ions were not added to the reaction mixture in the presence of fumarate or succinate. A 2 g. mitochondrial pellet containing 30 mg. total nitrogen was ashed and found to contain less than 10 µg. magnesium oxide by spectrographical analysis carried out by Mr A.B. Calder at the Edinburgh and East of Scotland College of Agriculture. The usual reaction mixtures containing 1.4 mg. total nitrogen might be expected to contain less than 3.8×10^{-6} mM magnesium ion. A requirement for magnesium is thus shown, but the binding of magnesium by the mitochondrial enzyme system enables it to carry on some 11β-hydroxylation of DOC in the absence of added magnesium ion.

5) The function of fumarate in the enzymic 11β-hydroxylation of DOC.

Introduction

Recently, Hayano & Dorfman (1953) have claimed that fumarate is absolutely required for the 11β-hydroxylation reaction. This requirement has been found using adrenal homogenate residues prepared by centrifuging homogenates at 5000 g. Previously the same workers had shown that,using whole homogenates of ox adrenal, several citric acid cycle intermediates functioned as activators of 11β-hydroxylation. This observation was also made by Kahnt & Wettstein (1951). The results of experiments reported now show that all members of the citric acid cycle tried activate DOC 11β-hydroxylation. Fumarate is a good activator. The possibility existed that other citric acid cycle intermediates activated DOC 11β-hydroxylation by being themselves converted to fumarate. Experiments were therefore carried out to investigate the requirement for fumarate for 11β-hydroxylation.

a) Effect of different concentrations of fumarate and succinate on 11β-hydroxylation of DOC.

If fumarate functions as a hydrogen acceptor in the 11β-hydroxylation reaction according to the equation

fumarate + 2H $\rightleftharpoons$ succinate

an adequate concentration of succinate might be expected to inhibit DOC 11β-hydroxylation. Also it might be expected that a high concentration of fumarate might permit 11β-hydroxylation to proceed in the absence of oxygen.

The effect of increasing the succinate concentration is shown in Fig. 9(a). Incubation flasks contained the usual reaction mixture and the concentration of succinate shown. The results show that the effect of increasing succinate concentration is to increase the DOC 11β-hydroxylation.

To test the second possibility, anaerobic experiments with varying concentrations of fumarate were performed in evacuated Thunberg tubes. In these experiments the Thunberg tubes were charged with the usual reaction mixture and the concentration

a) Effect of different concentrations of fumarate and succinate on 11β-hydroxylation of DOC.

If fumarate functions as a hydrogen acceptor in the 11β-hydroxylation reaction according to the equation

$$\text{fumarate} + 2H \rightleftharpoons \text{succinate}$$

an adequate concentration of succinate might be expected to inhibit DOC 11β-hydroxylation. Also it might be expected that a high concentration of fumarate might permit 11β-hydroxylation to proceed in the absence of oxygen.

The effect of increasing the succinate concentration is shown in Fig. 9(a). Incubation flasks contained the usual reaction mixture and the concentration of succinate shown. The results show that the effect of increasing succinate concentration is to increase the DOC 11β-hydroxylation.

To test the second possibility, anaerobic experiments with varying concentrations of fumarate were performed in evacuated Thunberg tubes. In these experiments the Thunberg tubes were charged with the usual reaction mixture and the concentration

of fumarate shown. The DOC was added last and the tubes were then immediately evacuated for 5 minutes using an oil-pump. Prior to addition of mitochondrial suspension and DOC the tubes were kept for at least 10 minutes in crushed ice to prevent any DOC metabolism occurring before anaerobic conditions were obtained. During the evacuation the tubes were kept in crushed ice. The results are shown in Fig. 9(b) and indicate that no appreciable 11β-hydroxylation occurs in the absence of oxygen with concentrations of fumarate up to 10 mM. Aerobic incubations with similar fumarate concentrations were also tried, these tubes being opened to the air after evacuation to check the effect of evacuation on the mitochondrial enzymes. The extensive 11β-hydroxylation of DOC in these control aerobic tubes demonstrates that the mitochondrial enzymes involved are undamaged by evacuation. This experiment has thus shown a requirement for oxygen for 11β-hydroxylation.

b) <u>The effect of malonate and the activation of DOC 11β-hydroxylation by the oxidation of a-oxoglutarate to succinate</u>.

The results so far obtained suggest that the concurrent oxidation of some citric acid cycle intermediates will enable 11β-hydroxylation of DOC to proceed. These experiments, however, have not eliminated the possibility of some special catalytic role for fumarate in 11β-hydroxylation since this substance will be formed from other intermediates by the operation of the citric acid cycle. The formation of fumarate may be inhibited by the inhibition by malonate of succinic dehydrogenase. Further, it appeared to be of interest to determine whether the 'one-step' reaction

a-oxoglutarate ⟶ succinate

could activate DOC 11β-hydroxylation. Previous experiments have suggested a connection between oxidations in the citric acid cycle and DOC 11β-hydroxylation. The ability of this 'one-step' oxidation to activate 11β-hydroxylation was therefore investigated.

Metabolism of DOC was studied in the presence of succinate and malonate and also with α-oxoglutarate and malonate. In both cases fumarate formation should be inhibited by the malonate.

RESULTS

100 mg. wet weight mitochondria (about 1.4 mg. total nitrogen). Standard reaction mixture plus 0.5 mM-ATP. Succinate and α-oxoglutarate at a concentration of 2 mM. Incubation in air, 1 hour at 37°.

	DOC recovered		Corticosterone recovered	
	(µg.)	(%)	(µg.)	(%)
After incubation				
Succinate	35	7	443	92
	45	9	439	91
Succinate plus	410	91	24	5
5 mM malonate	410	91	19	4
Succinate plus	415	92	28	6
10 mM malonate	415	92	18	4
α-Oxoglutarate	0	0	466	97
	5	1	463	96
α-Oxoglutarate	20	4	461	96
plus 5 mM malonate	10	2	461	96
α-Oxoglutarate	0	0	461	96
plus 10 mM malonate	5	1	468	97
Extraction control				
	450	98	465	96.5
	455	99	462	96
DOC added				
	460			

Conclusion

The results obtained indicate that the activation of 11β-hydroxylation by 2 mM-succinate is almost completely abolished by 10 mM-malonate. With succinate oxidation blocked in this way (this was checked by oxygen-uptake experiments) it can be seen that the 'one-step' oxidation of α-oxoglutarate to succinate enabled considerable DOC 11β-hydroxylation to proceed. The oxygen-uptake with α-oxoglutarate and 10 mM-malonate is quite considerable and such oxidation will allow DOC 11β-hydroxylation to occur concurrently. This experiment appears to exclude any specific role for fumarate in DOC 11β-hydroxylation by ox-adrenocortical mitochondria.

This last experiment was considered to be an extremely important one in augmenting the evidence that the citric acid cycle acids, by their oxidation, play some role in allowing 11β-hydroxylation to occur. This experiment has therefore been repeated five times with identical results each time. A particularly careful check was made on the identification of the metabolic product of DOC metabolism under these conditions. All the evidence pointed to it being solely corticosterone.

6) The effect of 2:4-dinitrophenol (DNP) on DOC 11β-hydroxylation.

The main object from the outset of this investigation has been to attempt to show some relationship between oxidative phosphorylation and 11β-hydroxylation; Kahnt & Wettstein (1951) having already suggested that processes of oxidative phosphorylation might be involved. Loomis & Lipmann (1948) showed that DNP in low concentrations stimulated respiration but simultaneously uncoupled oxidative phosphorylation. The effect of this substance on DOC 11β-hydroxylation was therefore investigated as it was thought that oxidative phosphorylation reactions involving the intermediates of the citric acid cycle were necessary for 11β-hydroxylation.

The effect of varying concentrations of DNP on DOC 11β-hydroxylation was tried. At the same time oxygen-consumption measurements were done in order to check the effect of the DNP concentrations on oxygen-consumption.

RESULTS

100 mg. wet weight mitochondria (about 1.4 mg. total nitrogen). Standard reaction mixture. DNP solutions pH 7.4. Incubation in air, 1 hour at 37°.

	DOC recovered (µg.)	DOC recovered (%)	Q_{O_2}
After incubation			
With 2 mM-fumarate	303	57	6.0
	297	56	
With 2 mM-fumarate plus 0.2 mM-DNP	467	88	6.1
	444	84	
With 2 mM-fumarate plus 0.5 mM-DNP	468	88	4.3
	482	91	

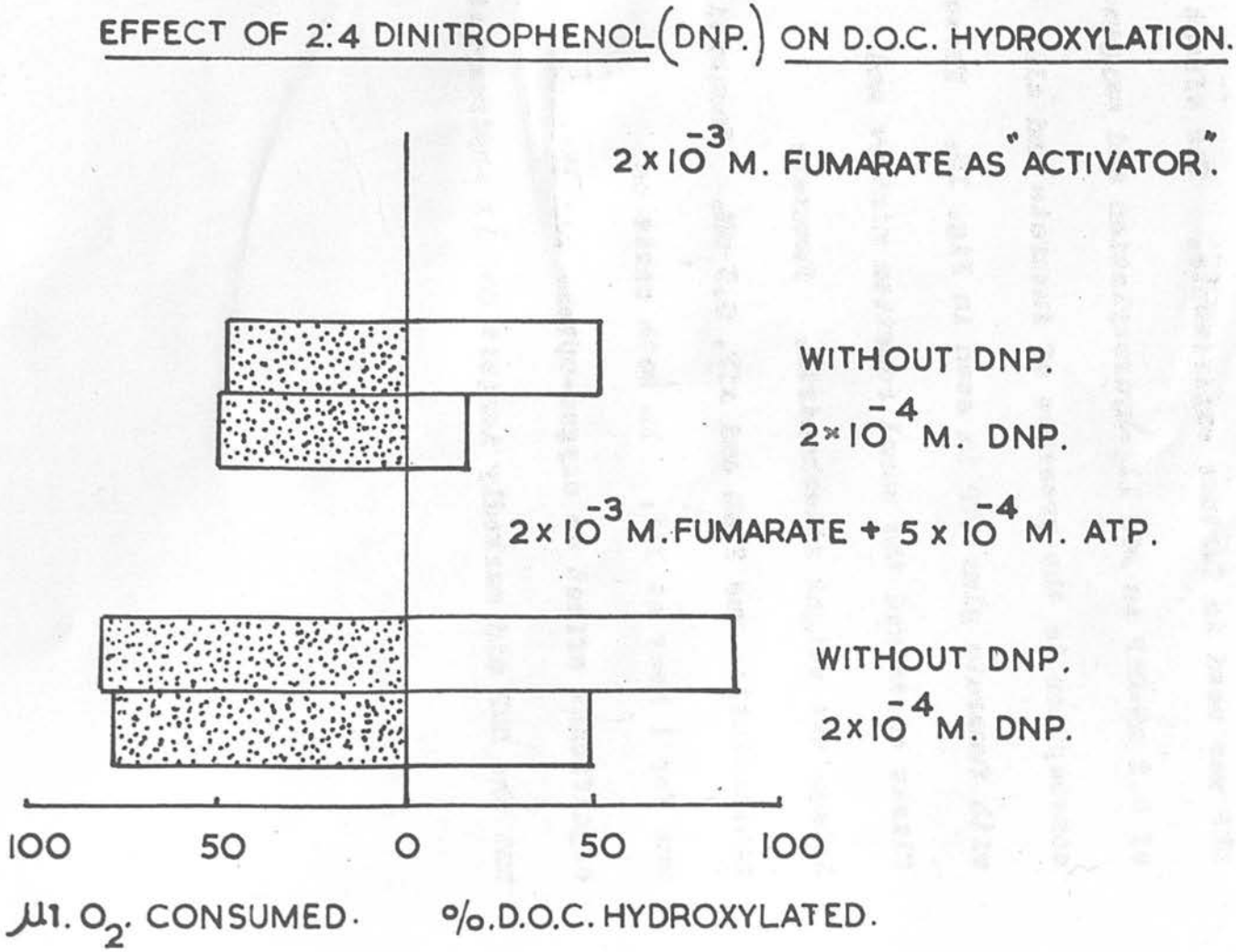

EFFECT OF 2:4 DINITROPHENOL (DNP.) ON D.O.C. HYDROXYLATION.
2 x 10⁻³ M. FUMARATE AS "ACTIVATOR".
WITHOUT DNP.
2 x 10⁻⁴ M. DNP.
2 x 10⁻³ M. FUMARATE + 5 x 10⁻⁴ M. ATP.
WITHOUT DNP.
2 x 10⁻⁴ M. DNP.
100
50
0
50
100
µl. O2. CONSUMED.
%. D.O.C. HYDROXYLATED.

Fig. 10

In the above experiment and other confirmatory ones, 0.5 mM-DNP although markedly inhibiting DOC 11β-hydroxylation also inhibited oxygen-consumption. Previous experiments having suggested a relationship between 11β-hydroxylation and the oxidation of citric acid cycle acids, the effect of the high concentration of DNP was, as expected, an inhibitory one. However, in the above experiment DNP at a concentration of 0.2 mM again inhibited DOC 11β-hydroxylation considerably, without inhibiting oxygen-consumption. This low concentration of DNP was used in further experiments. The effect of 0.2 mM-DNP on DOC 11β-hydroxylation and oxygen-consumption in the presence of fumarate and also with fumarate plus ATP is seen in Fig. 10. These flasks contained the usual reaction mixture and 100 mg. wet weight mitochondria. Fumarate concentration was 2 mM, and ATP 0.5 mM. Incubation was for 1 hour at 37°. In both cases no significant effect on oxygen-uptake was obtained, but the DNP did markedly inhibit DOC 11β-hydroxylation.

7) The effect of cyanide on DOC 11β-hydroxylation.

It would appear that DOC 11β-hydroxylation is somehow dependant on oxidation reactions in the citric acid cycle. These oxidations, catalyzed by dehydrogenases, depend upon a normally functioning hydrogen and electron transporting mechanism. Cyanide is a powerful inhibitor of cytochrome oxidase, the last stage in the electron transporting mechanism, and therefore of oxidations in the citric acid cycle. The effect of this substance upon DOC 11β-hydroxylation was therefore investigated.

RESULTS

100 mg. wet weight mitochondria/flask.

Standard reaction mixture. Cyanide concentration 10 mM. Fumarate concentration 2 mM.

	Time (min.)	DOC recovered (µg.)	(%)
After incubation			
With fumarate	15	265	53
	15	260	52
With fumarate plus cyanide	15	360	72
	15	345	69
With fumarate	30	90	18
	30	115	23
With fumarate plus cyanide	30	290	58
	30	320	64
With fumarate	60	15	3
	60	5	1
With fumarate plus cyanide	60	225	45
	60	222	44
Extraction control		465	93
DOC added		500	

Conclusion

Inhibition of DOC 11β-hydroxylation in the presence of cyanide has been demonstrated again stressing the importance of oxidative reactions required for 11β-hydroxylation.

SECTION II

THE METABOLISM OF 11-DEOXYCORTICOSTERONE BY OX-ADRENOCORTICAL HOMOGENATES

1) The effect of citrate on DOC 11β-hydroxylation by homogenates.

Because of the poor effect of added citrate on DOC 11β-hydroxylation various explanations were sought. As previously reported, increasing the magnesium ion concentration has no effect on 11β-hydroxylation in the presence of citrate. The poor oxygen-consumption obtained with citrate added to adrenocortical mitochondria suggested a possible lack of isocitric dehydrogenase in this preparation. On the other hand, poor citrate oxidation could be due to partial impermeability of the mitochondria to citrate as reported by Schneider (1953).

The effect of citrate on DOC 11β-hydroxylation by ox-adrenocortical homogenates was tried. These homogenates were prepared in 1.15% KCl by the usual homogenization technique. Steroid analyses were carried out by the usual method.

RESULTS

250 mg. homogenate per flask. Succinate and citrate concentrations 2 mM. Standard reaction medium. Incubation, 1 hour at 37°.

	DOC recovered	
	(µg.)	(%)
After incubation		
With succinate	15	3
	10	2
With citrate	50	11
	40	9
Extraction control		
	450	99
DOC added		
	454	

Conclusion

Citrate can function efficiently as an activator of DOC 11β-hydroxylation in adrenocortical homogenates. Hogeboom & Schneider (1950) have evidence that some isocitric dehydrogenase is lost in the isolation of mitochondria, and this seems a likely explanation of the poor 11β-hydroxylation of DOC in mitochondria with added citrate. In homogenates this would not apply.

Paper chromatograms were run on the extracts from these incubations and they showed corticosterone as the principal metabolite of DOC with only traces of more polar products.

2) The effect of 2:4-dinitrophenol (DNP) on DOC 11β-hydroxylation by homogenates.

The tissue to steroid ratio was reduced in this experiment in order to test the effect of DNP on DOC metabolism.

RESULTS

150 mg. homogenate per flask. Standard reaction medium. DNP concentration of 0.2 mM. 'Activator' concentration 2 mM. Incubation for 45 min. at 37°.

	DOC recovered (μg.)	(%)
After incubation		
No activator	450	94
No activator plus DNP	460	96
Succinate	40	8
Succinate plus DNP	320	67
Citrate	260	54
Citrate plus DNP	400	84
Extraction control	460	96
DOC added	480	

Conclusion

This low concentration of DNP has markedly inhibited DOC 11β-hydroxylation, reproducing the effect found with adrenocortical mitochondria.

SECTION III

THE METABOLISM OF 11-DEOXYCORTICOSTERONE BY OX LIVER MITOCHONDRIA.

1) Introduction

Conflicting reports have been made concerning the ability of tissues other than the adrenal to effect hydroxylations. Hechter et al. (1951) reported that neither rat liver nor human placenta could convert DOC to corticosterone. Seneca et al. (1950) claimed conversion of DOC to cortisone by liver, kidney, testes and ovarian slices, in addition to adrenal slices. Kahnt & Wettstein (1951) demonstrated hydroxylation activity with homogenates of liver and kidney, but none with brain or heart. Using rabbit and ox liver these workers claim conversion of 17α-hydroxy-11-deoxycorticosterone to 17α-hydroxycorticosterone amounting to only 1-5%. The metabolic product was characterized by paper chromatography including runs with mixtures of metabolite and 17α-hydroxycorticosterone. Axelrod & Miller (1953) reported 11β-hydroxylation of steroids on perfusion through rat liver. Hayano, Wiener & Lindberg (1953) have reported the presence of a 6β-hydroxylating enzyme in corpus luteum. Hubener & Amelung (1953) reported confirmation of the work

done by Kahnt & Wettstein (1951) with ox-liver in that they reported 11β-hydroxylation of 17α-hydroxy-11-deoxycorticosterone. Their evidence was also based on chromatographic work and this seems to have been suspect as recently Hubener & Schmidt Thome (1954) have reported that what was thought to be 17α-hydroxy-corticosterone was, in fact, pregn-4-ene-3-one-17α:20β:21-triol. They now suggest that 11β-hydroxylation does not occur in any organ other than the adrenal.

2) Incubation of DOC with ox liver mitochondria.

Ox liver mitochondria were prepared by the usual procedure.

RESULTS

100 mg. wet weight ox liver mitochondria per flask. Standard reaction mixture plus 0.5 mM-ATP. Fumarate concentration 10 mM. Incubation in air, 1 hour at 37°.

	DOC recovered	
	(µg.)	(%)
After incubation		
	496	94
	490	93
	485	92
Extraction control		
	500	95
DOC added		
	526	

Conclusion

No appreciable metabolism of DOC has occurred under conditions which with adrenal tissue would give 100% 11β-hydroxylation of DOC. DOC is converted to several reduction products by rat liver homogenates (Schneider, 1952) and if this is also the case in ox liver, then the enzymes involved are probably not present in the mitochondrial fraction.

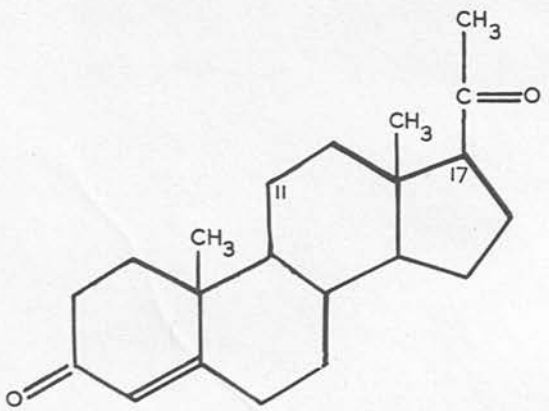
SECTION IV

THE METABOLISM OF PROGESTERONE BY OX-ADRENOCORTICAL MITOCHONDRIA

Introduction

On the basis of the results of experiments in which various steroids were perfused through ox-adrenal glands, Hechter *et al.* (1951) were able to present a tentative scheme of adrenocortical hormone biogenesis. Progesterone is postulated as a key intermediate in this scheme (Fig. 11) as perfusion of this steroid gave the following products:- 17α-hydroxyprogesterone (3-7%), 17α-hydroxy-corticosterone (10-12%), corticosterone (1.2 - 2.4%) and 11β-hydroxyprogesterone (less than 1%). The figures in parenthesis indicate the amounts of steroids recovered. Using ox-adrenal homogenates prepared in saline, Hayano & Dorfman (1952) confirmed the ability of the adrenal gland to form corticosterone from progesterone. The same workers (Hayano & Dorfman, 1953) in subsequent experiments using 'residue preparations of twice-washed ox-adrenal homogenates obtained at 5000 *g*' failed to convert progesterone to 11β-hydroxyprogesterone using conditions which permitted 11β-hydroxylation of DOC to take place in good yield. They attributed this failure to the absence of a hydroxyl group at the 21-position in the progesterone molecule. Similarly 17α-hydroxy-progesterone, which with whole homogenates was

SCHEME OF "CORTICOSTEROIDOGENESIS" —— O.HECHTER. et al.



PROGESTERONE ----------> 11β-HYDROXY PROGESTERONE. (< 1%.)

17α-HYDROXY PROGESTERONE (3–7%.)

17α-HYDROXY. D.O.C.

17α-HYDROXY CORTICOSTERONE (F) (10–12%.)

17α-HYDROXY, 11-DEHYDROCORTICOSTERONE. (E).

11-DEOXYCORTICOSTERONE (D.O.C.)

CORTICOSTERONE. (1·2 — 2·4%.)

11-DEHYDROCORTICOSTERONE.

Fig. 11

Introduction

On the basis of the results of experiments in which various steroids were perfused through ox-adrenal glands, Hechter et al. (1951) were able to present a tentative scheme of adrenocortical hormone biogenesis. Progesterone is postulated as a key intermediate in this scheme (Fig. 11) as perfusion of this steroid gave the following products:- 17α-hydroxyprogesterone (3-7%), 17α-hydroxy-corticosterone (10-12%), corticosterone (1.2 - 2.4%) and 11β-hydroxyprogesterone (less than 1%). The figures in parenthesis indicate the amounts of steroids recovered. Using ox-adrenal homogenates prepared in saline, Hayano & Dorfman (1952) confirmed the ability of the adrenal gland to form corticosterone from progesterone. The same workers (Hayano & Dorfman, 1953) in subsequent experiments using 'residue preparations of twice-washed ox-adrenal homogenates obtained at 5000 g' failed to convert progesterone to 11β-hydroxyprogesterone using conditions which permitted 11β-hydroxylation of DOC to take place in good yield. They attributed this failure to the absence of a hydroxyl group at the 21-position in the progesterone molecule. Similarly 17α-hydroxy-progesterone, which with whole homogenates was

converted to 17α-hydroxycorticosterone, was not further metabolized by this homogenate residue preparation. In the scheme proposed by Hechter et al. (1951) progesterone is converted by 21-hydroxylation to DOC prior to 11β-hydroxylation, also 17α-hydroxyprogesterone is converted again by 21-hydroxylation to 17α-hydroxy-11-deoxycorticosterone prior to 11β-hydroxylation. The 21-hydroxylating enzyme system which Plager & Samuels (1953) have shown to be present in the supernatant fraction of a homogenate centrifuged at 20000 g would presumably have been removed on washing the homogenate residue preparation used by Hayano & Dorfman. From their results these authors argue that progesterone has to be 21-hydroxylated prior to 11β-hydroxylation.

Previous experiments, now reported, on the 11β-hydroxylation of DOC by ox-adrenocortical mitochondria have suggested that this requires concurrent oxidative phosphorylation for which citric acid cycle intermediates are oxidizable substrates. The observation by Grant & Taylor (1952) that progesterone has a greater inhibitory effect than DOC on certain reactions of the citric acid cycle in rat liver mitochondria, suggested that similar effects in adrenocortical mitochondria might explain the failure of Hayano & Dorfman (1953) to obtain 11β-hydroxylation

of progesterone with adrenal homogenate residue preparations. Consequently it was decided to study conditions for the 11β-hydroxylation of progesterone by ox-adrenocortical mitochondria with particular reference to the influence of progesterone on reactions supporting the 11β-hydroxylation reaction.

1) Influence of progesterone and DOC on reactions of the citric acid cycle supporting 11β-hydroxylation.

a) Effect of progesterone and DOC on citrate metabolism.

The influence of these steroids on citrate metabolism was investigated by measuring the amount of citrate accumulating in reaction mixtures on incubation with pyruvate and L-malate. Using rat liver mitochondria Grant & Taylor (1952) demonstrated accumulation of citrate on incubation with pyruvate and L-malate. This accumulation is probably due to the loss of most of the isocitric dehydrogenase activity in the preparation of the mitochondria. In incubation flasks in which citrate was to be determined, incubations were terminated by addition of 3 ml. 30% (w/v) trichloracetic acid. After centrifugation, citrate was determined in a portion of the supernatant by the method of Taylor (1953). Citrate determinations were carried out by Dr Grant and D.W. Davidson in this Department.

The effect of progesterone and DOC on citrate accumulation from pyruvate and L-malate was studied using ox-adrenocortical mitochondria. The effect of varying the mitochondrial concentration on citrate accumulation was also investigated.

RESULTS

500 µg. steroids added in 0.04 ml. propylene glycol. Standard reaction mixture supplemented with 16 mM-potassium pyruvate and 16 mM-potassium L-malate. Incubation in air, 1 hour at 37°.

Wet weight mitochondria (mg.)	Steroid added	Citrate accumulated (µg.)
50	No steroid	382
	Progesterone	55
	DOC	245
100	No steroid	458
	Progesterone	120
	DOC	450
200	No steroid	475
	Progesterone	110
	DOC	590

Conclusion

The results of this experiment show that much less citrate accumulates in the presence of progesterone than with an equivalent amount of DOC at all enzyme concentrations. DOC inhibits citrate accumulation at low enzyme concentrations but stimulates this accumulation at higher concentrations. By increasing the mitochondrial concentration the inhibition by progesterone of citrate accumulation decreases, but even using 200 mg. wet weight mitochondria per flask, progesterone is a very effective inhibitor.

The greater inhibition by progesterone may be due to more powerful inhibition by this steroid of reactions converting pyruvate and L-malate to citrate or, greater stimulation, or less inhibition of citrate oxidation than DOC. Therefore the direct effect of progesterone and DOC on citrate oxidation by ox-adrenocortical mitochondria was investigated. In these experiments citrate was added to the standard reaction mixture and its metabolism determined by citrate estimations following incubation in the presence and absence of progesterone and DOC.

RESULTS

500 µg. steroids added in 0.04 ml. propylene glycol. Standard reaction mixture supplemented with 1 mM-citrate (576 µg. citric acid/flask). 100 mg. wet weight mitochondria/flask. Incubation in air, 1 hour at 37°.

Steroid added	Citrate oxidized (µg.)
No steroid	315
Progesterone	103
DOC	92

Conclusion

Both steroids inhibit citrate oxidation to about the same extent. The small difference is insufficient to explain the marked difference between these steroids with regard to inhibition of citrate synthesis from pyruvate and L-malate. Progesterone must therefore be inhibiting reactions involved in the synthesis of citrate from pyruvate and L-malate. This inhibition could be at the malic dehydrogenase, pyruvic oxidase, or condensing-enzyme steps.

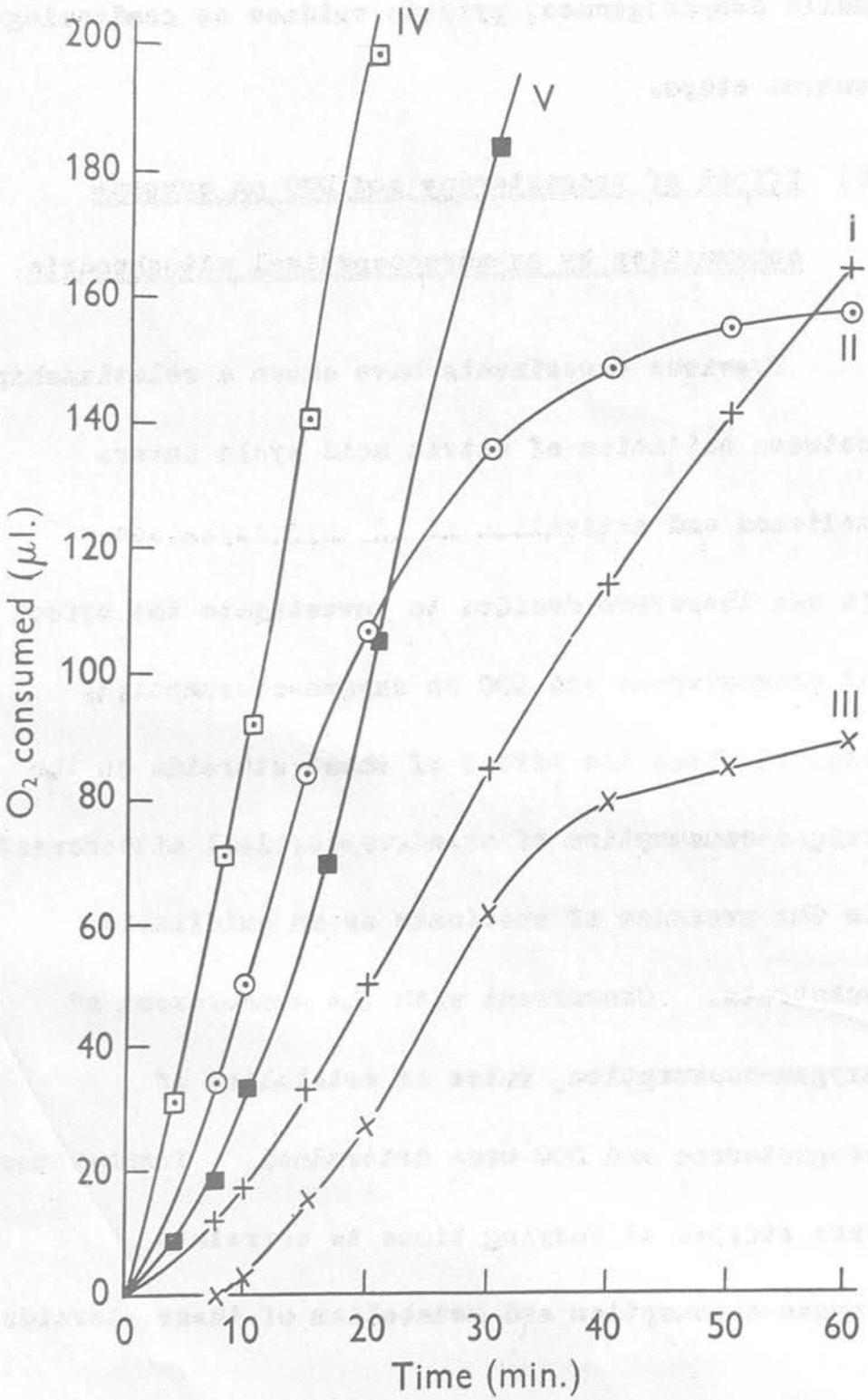


Fig.12 Effect of progesterone and DOC on succinate oxidation

I 100mg. wet weight mitochondria, 2mM-succinate.

II As I plus DOC. III As I plus progesterone.

IV 200mg. wet weight mitochondria, plus 10mM-succinate.

V As IV plus progesterone.

b) Effect of progesterone and DOC on oxygen-consumption by ox-adrenocortical mitochondria.

Previous experiments have shown a relationship between oxidation of citric acid cycle intermediates and activation of 11β-hydroxylation. It was therefore decided to investigate the effect of progesterone and DOC on oxygen-consumption. Fig. 12 shows the effect of these steroids on the oxygen-consumption of ox-adrenocortical mitochondria in the presence of succinate as an oxidizable substrate. Concurrent with the measurement of oxygen-consumption, rates of metabolism of progesterone and DOC were determined. Incubations were stopped at varying times to correlate oxygen-consumption and metabolism of these steroids.

RESULTS

100 mg. wet weight mitochondria per flask.
Standard reaction mixture plus 2 mM-succinate.
Incubation in air, 1 hour at 37°.

Time (min.)	DOC recovered (µg.)	(%)	Progesterone recovered (µg.)	(%)
5	350	58	680	96.5
	370	61	690	98
10	70	12	600	85
	65	11	610	86.5
15	28	3	600	85
	40	6.5	600	85
30	5	1	605	86
	0	0	600	85
60	0	0	600	85
Extraction control	607	100	670	95
Steroid added	607		704	

Conclusion

These results show that added DOC is almost all metabolized in the first 10 minutes of incubation; a period during which there is marked stimulation of oxygen uptake as shown in Fig. 12. In contrast, added progesterone inhibits oxygen uptake particularly during the first 10 minutes and is not metabolized appreciably during this period.

c) The effect of preincubation of ox-adrenocortical mitochondria on DOC 11β-hydroxylation.

The results of the previous experiments suggested that the adrenocortical mitochondrial enzymes, directly or indirectly involved in steroid 11β-hydroxylation, may be inactivated in the presence of progesterone, during the initial 10 minute incubation period during which the mitochondria are not respiring. Preincubation of rat liver 'cyclophorase' preparation in the absence of oxidizable substrate is known to lead to inactivation of many citric acid cycle dehydrogenase systems (Heunnekens & Green, 1950). The ability of such pre-incubated preparations to catalyze oxidative phosphorylation is also lost. Thus, it was decided to investigate the effect of similar pre-incubation

on the efficiency of 11β-hydroxylation of DOC in ox-adrenocortical mitochondria. The effect of pre-incubation was compared with the effect of progesterone on DOC 11β-hydroxylation.

RESULTS

100 mg. wet weight mitochondria per flask. Standard reaction mixture in each flask. 500 µg. steroids added in 0.04 ml. propylene glycol. Incubations in air at 37°. The following additions were made to duplicate flasks, and incubation conditions were as stated.

Flask A: Progesterone, DOC and 2 mM-succinate were present from the start. Incubation was for 10 min.

Flask B: Progesterone and 2 mM-succinate were present from the start. After 20 min. pre-incubation, DOC was added and incubation continued for a further 10 min.

Flask C: After 20 min. pre-incubation, DOC and 2 mM-succinate were added and incubation continued for a further 10 min.

Flask D: 2 mM-succinate was present from the start. After 20 min. pre-incubation DOC was added and incubation continued for a further 10 min.

Flask E: DOC and 2 mM-succinate were present from the start. Incubation was for 10 min.

Flask	DOC recovered (µg.)	DOC recovered (%)	DOC hydroxylated (%)
A	493	91.5	8.5
	493	91.5	8.5
B	488	90	10
	473	88	12
C	458	85	15
	468	87	13
D	172	33	67
E	136	25	75
	119	22	78
Extraction control	540	100	

Conclusion

From the results above it is clear that pre-incubation without oxidizable substrate (succinate), or with succinate oxidation inhibited by progesterone, greatly reduces the 11β-hydroxylation of DOC added subsequently. Preincubation of the mitochondria in the presence of succinate apparently retains the activity of the 11β-hydroxylating enzyme system.

2) Determination of conditions suitable for the metabolism of progesterone by ox-adrenocortical mitochondria.

The results of the previous experiments suggest that progesterone may be inhibiting reactions supporting 11β-hydroxylation. The first of these experiments has shown that by increasing the mitochondrial concentration, progesterone inhibition of citrate synthesis could be decreased somewhat. The effect of varying the concentrations of progesterone, mitochondria and oxidizable substrate (succinate) on progesterone metabolism was therefore investigated.

RESULTS

Standard reaction mixture supplemented with concentrations of succinate, mitochondria and progesterone shown. Incubation in air, 1 hour at 37°.

	Succinate (mM)	Progesterone recovered (µg.)	Progesterone recovered (%)	Progesterone metabolized (%)
After incubation				
100 mg. mitochondria plus 520 µg. progesterone	2	480	86	14
		460	82	18
	5	460	82	18
		480	86	14
	10	480	86	14
		480	86	14
200 mg. mitochondria plus 520 µg. progesterone	2	420	75	25
		410	73	27
	5	310	55	45
		290	52	48
	10	250	48	52
		240	46	54
200 mg. mitochondria plus 260 µg. progesterone	10	98	36	64
		94	34	66
Extraction control		247	95	
		244	94	

Conclusion

By increasing the concentration of succinate to 10 mM, and by using 200 mg. wet weight mitochondria, metabolism of progesterone has been increased to about 50%. The effect of added progesterone on succinate oxidation under these conditions is seen in Fig. 12. Under these incubation conditions the inhibition of oxygen-consumption during the initial 10 minutes has been overcome. Oxygen-consumption during the whole of the incubation period is not markedly affected. By overcoming the initial inhibition of succinate oxidation, progesterone metabolism can take place. These experiments have pointed out the importance of considering the effect of the steroid on reactions supporting its own metabolism.

3) Investigation of the products of metabolism of progesterone by ox-adrenocortical mitochondria.

a) Introduction

In the small scale experiments described above it was seen that using 10 mM-succinate, 200 mg. wet weight mitochondria, and about 250 µg. progesterone in incubation mixtures results in about 65% metabolism of progesterone. It was decided to investigate the metabolic products of progesterone under these conditions. The difficulty in preparing large amounts of mitochondria from the cortical tissue of fresh ox adrenals limited the amount of progesterone which could be incubated at any one time. From 15 g. adrenocortical tissue about 2 g. wet weight mitochondria are obtained, and by fractionating two 15 g. portions each day, 4 g. mitochondria were prepared. 5 mg. samples of progesterone in 0.4 ml. propylene glycol were incubated on each of 9 days with 4 g. mitochondria in 60 ml. of the standard reaction mixture containing 10 mM-succinate. This volume was distributed in a number of 100 ml. conical flasks which were shaken in air for 1 hour at 37°.

Progesterone determinations on small portions of this mixture after incubation indicated that about 60% of the steroid had been metabolized. A portion of the residue from the benzene-$CHCl_3$ extract prepared for the progesterone determinations was chromatographed on a 10 cm. Celite partition column, using the solvent system benzene-n-hexane (3:7, v/v) as mobile phase, and methanol:water (7:3, v/v) as stationary phase. This is the system used in DOC estimations. Observation of the elution of material absorbing selectively at 240 mμ indicated the presence of a substance slightly more polar than DOC. This material ('X') was spread over a cut from 30-45 ml. eluate whereas DOC is eluted from 18-26 ml. 'X' was always found after incubation of progesterone under these conditions. Periodate oxidation of 'X' yielded no formaldehyde as determined by the method of Daughaday, Jaffe & Williams (1948). This indicated that it was not a steroid having an α-ketol side chain at C-17. It did however have an α:β-unsaturated-3-ketone structure in the A ring as shown by its selective absorption of ultraviolet light at 240 mμ. 'X' was not present in 'blanks' prepared from adrenocortical mitochondria. No corticosterone could be detected in these extracts on chromatography on Celite partition columns. The chromatographic

behaviour of 'X', which appeared to be the main metabolite of progesterone incubation with adrenocortical mitochondria under these conditions, determined the procedure employed for its subsequent isolation and purification.

b) Experimental

i) Extraction

A total of 45 mg. progesterone were incubated with ox-adrenocortical mitochondria. Each day 5 mg. progesterone was incubated. Incubations were terminated by the addition of 5 volumes of chilled acetone and precipitated material was filtered off after standing for 2 hours at -20°. The precipitate and incubation flasks were washed twice with 5 volumes of chilled acetone. The filtrate and washings were evaporated *in vacuo* (water bath temperature was 50°) to an aqueous residue of about 50 ml. After dilution with an equal volume of water the aqueous residue was saturated with NaCl and extracted with 3 x 50 ml. ethyl acetate. The pooled ethyl acetate extracts were washed with 50 ml. 0.2 M-$NaHCO_3$, 50 ml. 0.2 N-HCl and finally with water until the washings were neutral. It was possible to carry out these operations quickly due to the rapid separation of

the ethyl acetate and aqueous phase so that the ester was in contact with acid or alkali for the shortest possible time. All washings were back-washed with one 50 ml. volume of ethyl acetate. After drying over anhydrous Na_2SO_4 the combined extracts were evaporated to dryness by distillation *in vacuo*. The resultant brown gum was partitioned in 100 ml. of a solvent system consisting of 20 ml. *n*-hexane and 80 ml. methanol:water (8:2, v/v). Much yellow-coloured material entered the hexane phase. The methanol: water phase after partition was separated off and evaporated *in vacuo* to an aqueous residue which was diluted to 50 ml. with water and extracted 3 x 50 ml. $CHCl_3$. The pooled $CHCl_3$ extracts were dried over anhydrous Na_2SO_4 and taken to dryness by distillation *in vacuo*.

The above procedure was done after each days incubations, the final residues being kept in a vacuum dessicator at 0°. Although this undoubtedly involved more work than if the reaction mixtures had been kept and extracted at the one time, the volumes involved were suitably small, and the incubations having been done in the morning it was quite convenient to finish the extractions in the afternoon. It was also perhaps safer to have the steroids extracted

from the reaction mixtures and in the dry, solid state with less chance of destruction occurring. Such was the purification obtained by the above extraction procedure that the final residue obtained each day showed selective absorption at 240 mμ when dissolved in ethanol. Most of the non-steroid material had been removed particularly at the n-hexane:methanol-water partition.

ii) Countercurrent distribution.

The residues from extracts of individual incubation mixtures were combined and submitted to a 6-stage countercurrent distribution with double withdrawal (Craig & Craig, 1950). This was carried out in separating funnels with the solvent system methanol:water (8:2, v/v), benzene-n-hexane (8:2, v/v), using 50 ml. of each phase. The partition coefficient (K) for progesterone in this system is 0.23, for DOC it is 1.0, and for 11β-hydroxyprogesterone it is 1.6. Fig. 13 shows the theoretical distribution for K = 0.23 and for K = 1.6 in such a distribution and also the actual distribution of isolated material absorbing selectively at 240 mμ. Analysis for material absorbing selectively at 240 mμ was carried out on known aliquots of the residues obtained from each funnel.

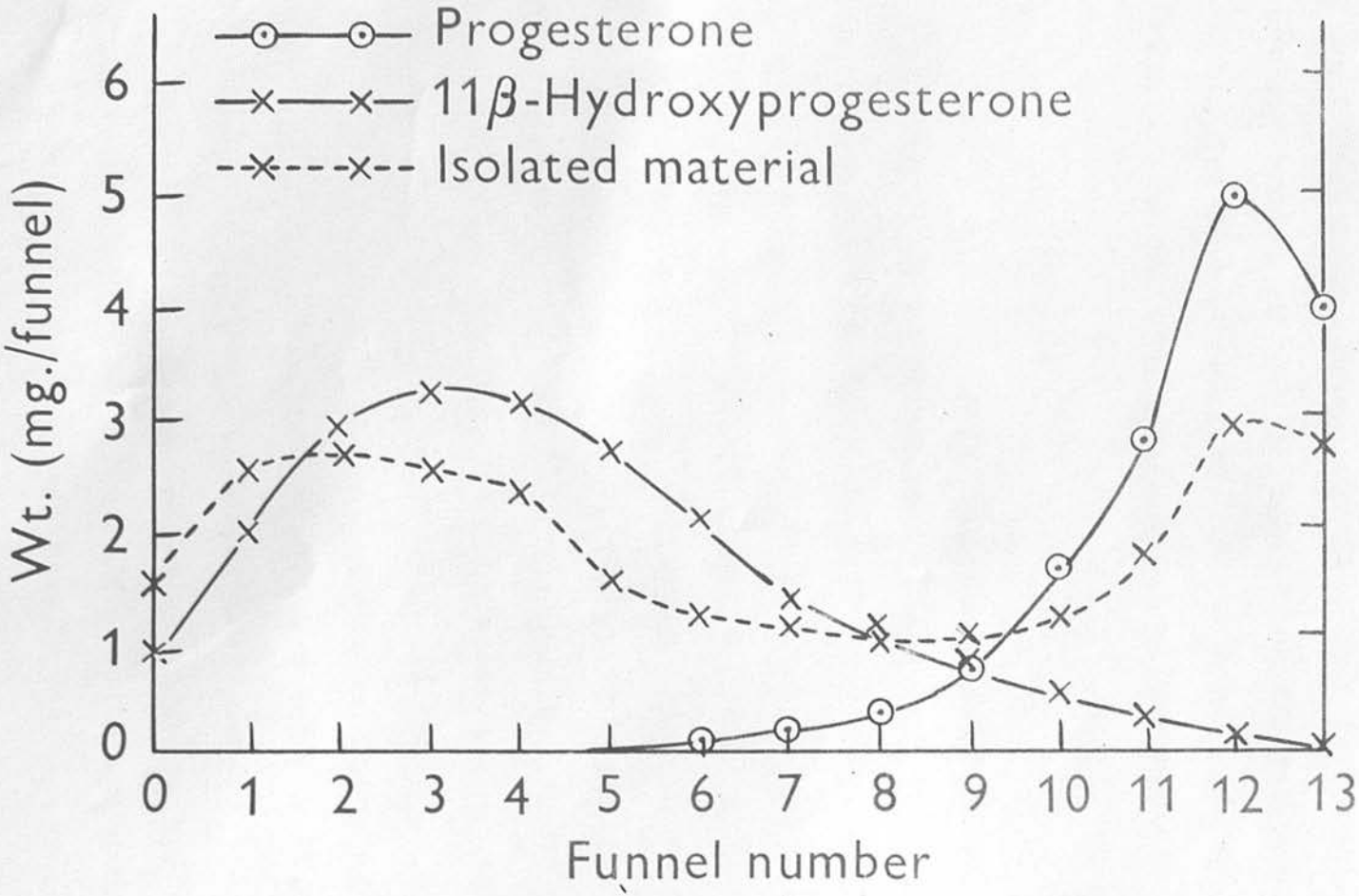

Progesterone
11β-Hydroxyprogesterone
Isolated material
Wt. (mg./funnel)
0
1
2
3
4
5
6
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Funnel number

FIG. 13.

The theoretical curves for K = 0.23 and K = 1.6 were calculated by first expanding the binomial

$$\left(\frac{1}{K+1} + \frac{K}{K+1} \right)^n$$

which gives the distribution of solute on completion of the normal distribution. The individual terms in this expansion may be expressed by the formula

$$T_{n.r} = \frac{n!}{r! \cdot n-r!} \cdot \left(\frac{1}{K+1} \right)^n \cdot K^r$$

where $T_{n.r}$ is the fraction of the original material present in the rth tube after n transfers. K is the ratio of solute in the lower phase to that in the upper phase. (Using separating funnels in this distribution it is the lower phase which is the mobile phase.) Using the above formula, the distribution of solutes having K = 0.23 and K = 1.6 after a 6-stage distribution could be calculated. Thereafter, the values of K are used to work out the effect of double withdrawal procedure on the distribution of these solutes.

From the distribution pattern of isolated material (Fig. 13) it can be seen that 60-70% metabolism has apparently occurred and that the

metabolic product or products are more polar than DOC behaving similarly to 11β-hydroxyprogesterone. Residues obtained on evaporating the contents of funnels 0-6 to dryness in vacuo were semi-crystalline. Their combined weight was 12.5 mg. Since adsorption chromatography had been successfully used by Reichstein & Fuchs (1940) for the isolation of 11β-hydroxy-progesterone, it was decided to employ this technique at this stage.

iii) Adsorption chromatography.

As a trial, a mixture of 600 µg. progesterone and 700 µg. 11β-hydroxyprogesterone was run on about 400 mg. alumina (Peter Spence & Sons Ltd., Widnes) activity II (Brockman & Schodder, 1941). The column consisted of the drawn out part of a 10 ml. bulb pipette with the bulb serving as a reservoir. Diameter of the column was about 4 mm. The chromatogram was developed as below, each fraction being 2 ml. Solvent ratios are all v/v.

Fraction No.	Solvent	D_{240}
1-3	Benzene-n-hexane (1:1)	4.0
4-6	Benzene-n-hexane (3:2)	0.45
7-9	Benzene-n-hexane (4:1)	0.12
10-17	Benzene-ether (95:5)	0.2
18-20	Benzene-ether (4:1)	2.0
21-23	Benzene-ether (3:2)	2.0
24-26	Benzene-ether (2:3)	0.35

A good separation of progesterone and 11β-hydroxyprogesterone has been obtained. Progesterone is eluted first, then 11β-hydroxyprogesterone.

The semi-crystalline material from tubes 0-6 was combined and chromatographed on a similar alumina column. Weight of alumina used was about 30 times the weight of material to be chromatographed. The combined residues were applied to the column in 3 ml. benzene-n-hexane (1:1, v/v). The chromatogram was developed as below. Each fraction was 3 ml. and all solvent ratios are v/v.

ABSORPTION CURVES OF THE SULPHURIC ACID CHROMOGENS OF PROGESTERONE METABOLITE AND 11β-HYDROXYPROGESTERONE.

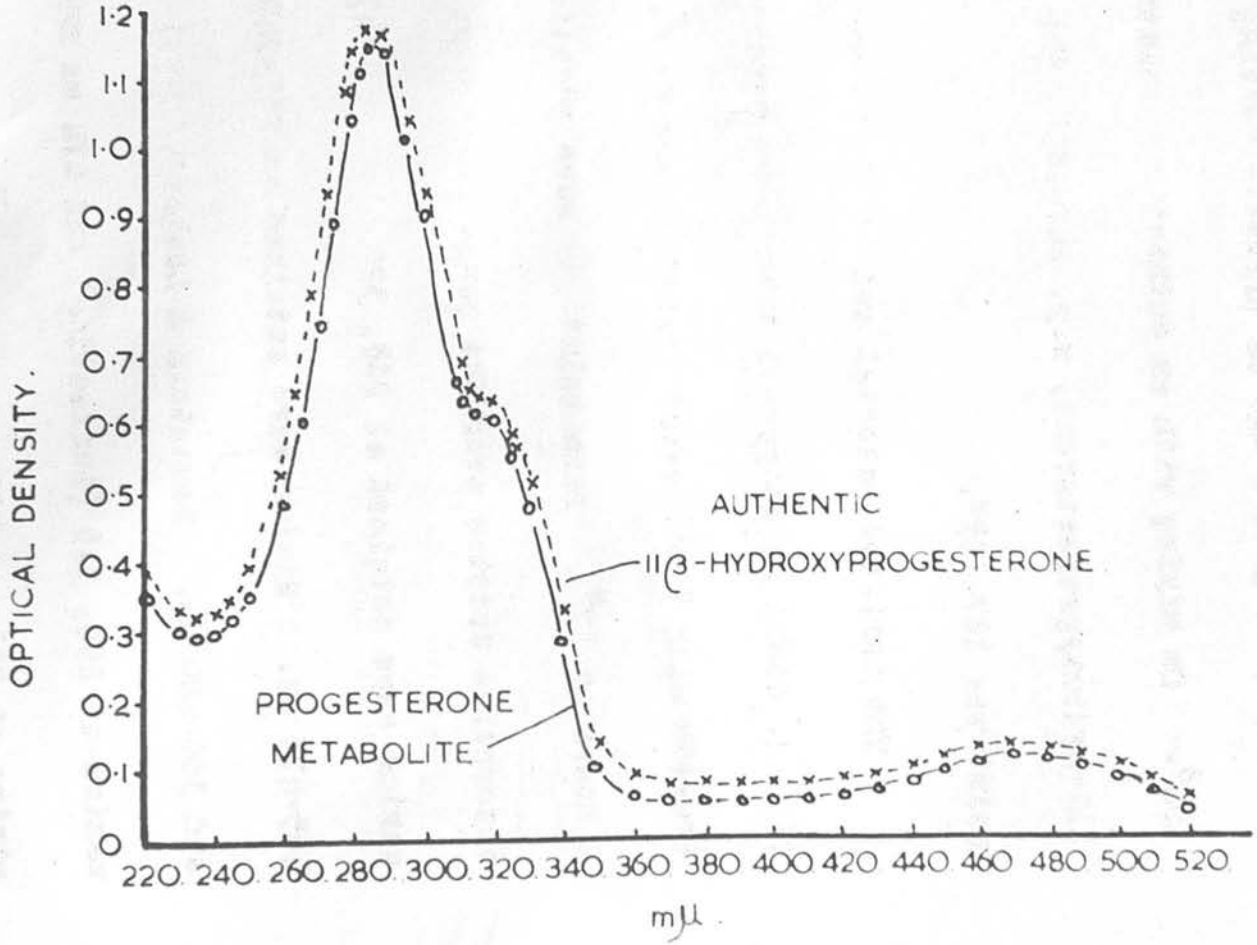


Fig. 14

Fraction no.	Solvent	Wt. (mg.)	Description
1-3	Benzene-n-hexane (1:1)	-	-
4-6	Benzene-n-hexane (3:2)	-	-
7-9	Benzene-n-hexane (4:1)	0.5	White crystals
10-12	Benzene	1.4	White crystals
13-15	Benzene-ether (9:1)	6.0	White crystals
16-18	Benzene-ether (4:1)	0.5	White crystals
19-21	Benzene-ether (3:2)	0.5	Amorphous solid

iv) Isolation of 11β-hydroxyprogesterone.

The white crystalline material from fractions 7-18 was recrystallized twice from benzene-n-hexane (1:1, v/v) to give 8 mg. of crystals melting at 189-191°. On mixing with an authentic specimen of 11β-hydroxyprogesterone, m.p. 187-189°, the melting point was 187-189°.

The isolated material and 11β-hydroxyprogesterone (70 µg. each) in stoppered tubes were separately treated with 5 ml. concentrated sulphuric acid for 2 hours at 17°. Both solutions gave identical absorption spectra over the range 220-520 mµ (Fig. 14). Maxima were obtained at 288, 320 (inflection) and 465-475 mµ. Minima were obtained at 235-240 mµ

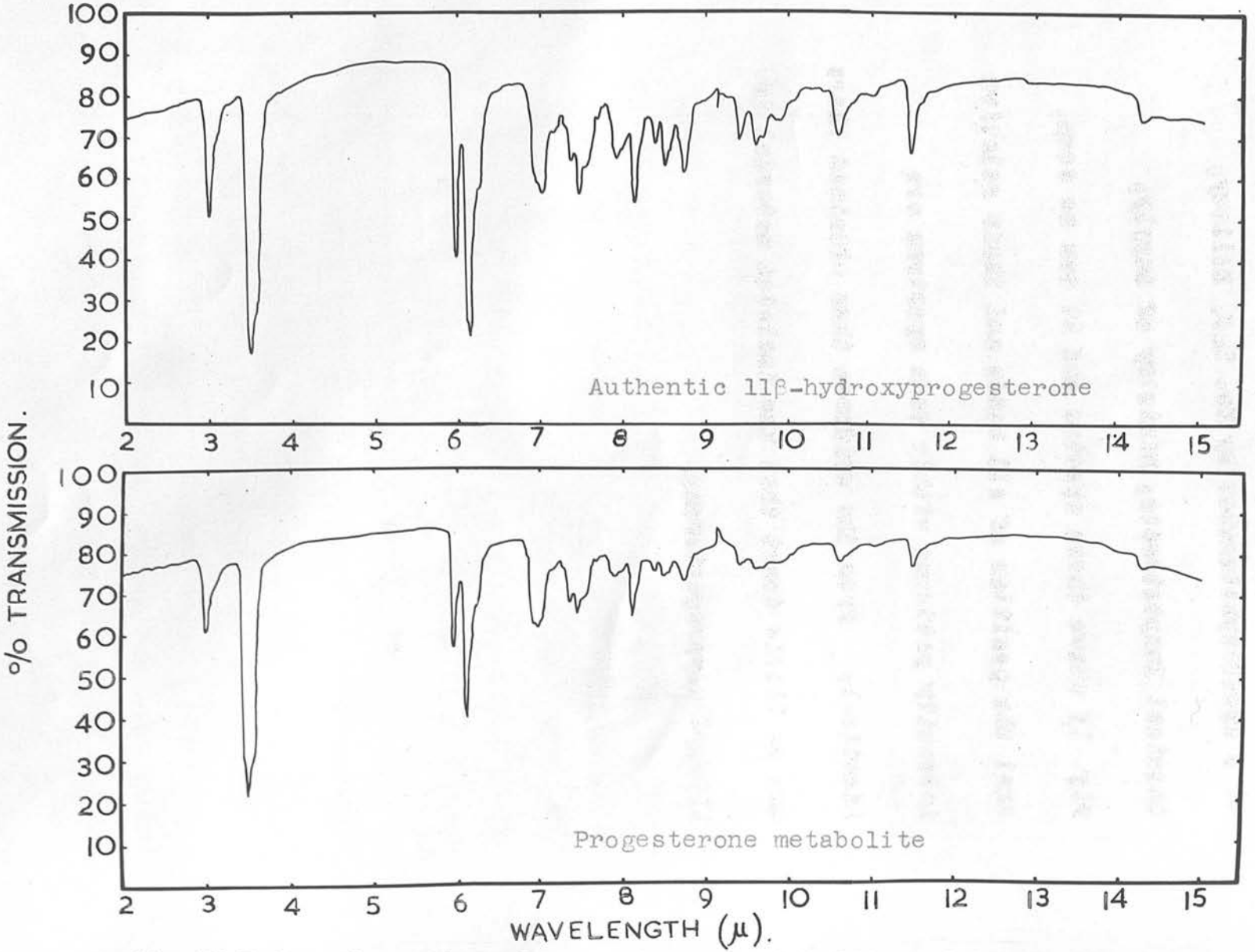


Fig.15 Infrared spectra of progesterone metabolite and 11β-hydroxyprogesterone

and 380-400 mμ. Bernstein & Leuhard (1953) report maxima at 290, 318 (inflection) and 474 mμ and minima at 231 and 380 mμ.

The infrared spectra of the isolated material melting at 189-191° and of the authentic 11β-hydroxyprogesterone were determined with mulls ground in paraffin in a double-beam Perkin-Elmer 21 B spectrophotometer by Dr L.J. Bellamy, Chemical Inspectorate, Ministry of Supply, London. Fig. 15 shows these spectra, and it can be seen that the position of all bands and their relative intensity patterns within each spectrum are identical. From the evidence thus obtained there can be little doubt that the isolated material is 11β-hydroxyprogesterone.

4) Recovery of progesterone and 11β-hydroxyprogesterone following incubation of progesterone with ox-adrenocortical mitochondria.

In these experiments progesterone was incubated with ox-adrenocortical mitochondria under the following conditions. About 500 µg. progesterone were incubated for 1 hour at 37° with 200 mg. wet weight mitochondria in the presence of 10 mM-succinate. Progesterone and 11β-hydroxyprogesterone in the reaction mixtures were determined by the methods already described. The results of a typical experiment are given below.

Progesterone recovered		11β-Hydroxyprogesterone recovered	
(μg.)	(μ moles)	(μg.)	(μ moles)
After incubation			
350	1.12	140	0.42
330	1.06	120	0.36
340	1.08	110	0.33
350	1.12	120	0.36
350	1.12	130	0.39
350	1.12	120	0.36
360	1.15	120	0.36
340	1.08	120	0.36
Extraction control			
500	1.6		

Summary

Progesterone added	Progesterone recovered	11β-Hydroxy-progesterone recovered	Total recovery	
(μ moles)	(μ moles)	(μ moles)	(μ moles)	(%)
1.60	1.1 ± 0.05	0.37 ± 0.04	1.47 ± 0.1	92 ± 6

Conclusions

These results indicate that the 11β-hydroxyprogesterone formed may account for the greater part of the progesterone metabolized by the mitochondria. Similar results to those reported have been obtained on many occasions. The results, however, suggest that progesterone may not be the sole metabolite as the total recoveries vary over the range 86-98%. Allowing for the extraction control only a trace of other metabolites could be present.

Attempts were made to obtain information on the nature of other metabolic products by paper chromatography of the benzene-$CHCl_3$ extract obtained in quantitative experiments. Portions of extracts containing 150 μg. material absorbing selectively at 240 mμ were employed. For enzyme blanks, similar portions were taken from extracts obtained after incubation of mitochondria without added steroids. For standards about 150 μg. each of progesterone, 11β-hydroxyprogesterone and DOC were added to separate portions of the blank extract. The chromatograms were simultaneously run in duplicate for 2 hours at 20° using system B_1 of Bush (1952). The dried papers were then sprayed with

(a) methanolic-NaOH reagent of Bush (1952) and

(b) the blue-tetrazolium reagent (Mader & Buck,1952). Chromatograms of extracts from progesterone incubation experiments showed spots corresponding to 11β-hydroxyprogesterone and progesterone when sprayed with the methanolic-NaOH reagent. Neither of these spots were shown up on spraying with the blue-tetrazolium reagent, and no spot corresponding to DOC was observed. With this reagent the presence of a trace of material more polar than DOC was indicated. This material behaved similarly to corticosterone on paper chromatograms. This more polar material was not observed on chromatograms of progesterone or with the mitochondrial blanks. Chromatograms were also run using the system A of Bush (1952) and again 11β-hydroxy-progesterone was found as the chief metabolite of progesterone. In this system DOC and 11β-hydroxy-progesterone are easily separated and these runs showed no DOC in the extracts.

Paper chromatography has confirmed that 11β-hydroxyprogesterone is the major metabolite of progesterone under these conditions.

SECTION V

GENERAL DISCUSSION

1) 11β-Hydroxylation of DOC by ox-adrenocortical mitochondria.

a) Analytical procedures

In this preliminary investigation of factors influencing the 11β-hydroxylation of DOC, use has been made of an excellent method for the determination of steroids in tissue extracts developed by Dr Taylor in this Department (1954). This method applied to DOC permits the recovery of 96 ± 1% of this steroid added to preparations of mitochondria. DOC and metabolic product are isolated by extraction and chromatographic techniques, and the recoveries of unchanged DOC and metabolic product measured by selective absorption at 240 mμ. Recoveries of unchanged DOC plus metabolic product are about 96%. The percentage recoveries are based on the actual amount of DOC added to the reaction mixture. The results compare favourably with analytical procedures previously reported. Hayano & Dorfman (1953) have made use of a method which permits the recovery of 86% DOC in extraction control experiments. After incubation of DOC with ox-adrenal homogenates these workers obtain recovery of DOC plus metabolic products of 80 ± 5%. They state that 'these figures were

taken as indices of 100 in the calculation of the percentage products formed'. The percentage recovery of metabolic products may be unduly high.

b) Enzyme preparations

In the work reported here, ox-adrenocortical mitochondria have been used as the source of 11β-hydroxylating enzyme. Incubation of DOC with such preparations under suitable conditions appears to yield largely corticosterone. The evidence obtained supports the view that DOC hydroxylation in adrenocortical mitochondria may be restricted to the 11β-position in the steroid nucleus. In support of this, Plager & Samuels (1953) have shown that hydroxylation of progesterone at the 17α- and 21-positions is catalyzed by enzymes in the supernatant fraction of adrenal homogenates centrifuged at 20000 g, and that further hydroxylation at the 11β-position is brought about by the mitochondrial fraction. The results obtained have confirmed the observation by Sweat (1951) that 11β-hydroxylation of steroids is catalyzed by enzymes in the particulate fraction of an adrenal homogenate.

Although the best methods available at present for the preparation of morphologically and biochemically intact mitochondria are far from ideal,

they are preferable to the procedures frequently used in preparation of adrenal tissue for steroid hydroxylation studies. Thus, for example, adrenal cell homogenates have been prepared in water or saline using the Waring Blendor (Hayano & Dorfman, 1953; Hayano, Dorfman & Yamada, 1951), which when used without speed reduction is known to break up mitochondria as well as whole cells (Hogeboom, 1951; Sourkar, Beinert, Fuld & Green, 1952). It is only possible to use the Waring Blendor at reduced speed for homogenization if the tissue is first disintegrated in a tissue press (Witter, Pories & Cottone, 1953). The mitochondria in preparations exposed to water or hypotonic media swell and sustain damage resulting in release of water-soluble proteins, nucleotides, enzymes and cofactors (Kennedy & Lehninger, 1949; Huennekens & Green, 1950; Schneider, 1949; Berthet, Berthet, Appelmans & de Duve, 1951). Incubation of whole homogenates results in extensive destruction of pyridine nucleotides by enzymes released from damaged cell components (Mann & Quastel, 1941). Saline-washed sediments are relatively free from soluble proteins, including released nucleotidases. Nevertheless, they contain a mixture of nuclei, intact mitochondria and mitochondria damaged by exposure to saline. Microsomes,

agglutinated by the electrolytes present (Schneider & Hogeboom, 1951), will also be in this homogenate residue. The use of such preparations in steroid 11β-hydroxylation studies (Hayano & Dorfman, 1953) must incur the danger of producing artificial steroid metabolic products by interruption of normal metabolic pathways in damaged cell components, or by abnormal interaction of enzymes from different cell components. Experiments with washed sediments can give no indication of the location of steroid-hydroxylating enzymes in the cell. Hayano & Dorfman (1954) have claimed that only by using such damaged preparations have they been able to demonstrate the function of fumarate as an essential cofactor for 11β-hydroxylation of DOC. However, these findings do not necessarily apply to whole cells or intact mitochondria, and certainly not to the intact gland.

The use of mitochondria isolated in sucrose solutions offers several advantages in the preliminary study of steroid hydroxylation. Whole cells, nuclei, and soluble proteins are eliminated so that their effect on steroid hydroxylation need not be considered. Contamination by microsomes is not extensive. Schneider (1953) has estimated this to be about 5% of the volume of the mitochondrial fraction. The reduction in the amount of cellular material used

simplifies steroid analysis. Isolated mitochondria retain many dehydrogenases with intact hydrogen and electron-transporting mechanisms and can catalyze oxidative-phosphorylation (Potter, Lyle & Schneider, 1951). Hayano & Dorfman (1953), using washed sediments, were unable to demonstrate 11β-hydroxylation of DOC in the absence of added magnesium. The 11β-hydroxylation of DOC now reported, observed in experiments in which added magnesium was not required, may reflect the intact nature of the mitochondria employed. With mitochondria, corticosterone has been obtained as the probable single product of DOC hydroxylation. Hayano & Dorfman (1953) obtained corticosterone and other unidentified products using washed adrenal sediments. It remains to be seen whether these other products are not artifacts produced by this type of enzyme preparation. Although most evidence points to the mitochondria as the site of the 11β-hydroxylating enzyme system, it may be that other cell components affect the steroid 11β-hydroxylation in the intact cell. In addition, Dorfman et al. (1953) reported that 11β-hydroxylation of DOC is catalyzed by ox-adrenal medulla as well as by the cortex. In all their experiments these workers have made use of homogenates of whole adrenals. On the other hand, Kahnt (1953)

reported that the adrenal medulla does not contain any appreciable amount of the 11β-hydroxylating enzyme, and that neither the medulla, nor pure adrenaline, have any specific action on the *in vitro* production of 17α-hydroxycorticosterone from 17α-hydroxy-11-deoxycorticosterone. The differences in the above reports may be due to the difficulty in separating medulla from cortex. That an enzyme may be present in adrenal medulla which will catalyze 11β-hydroxylation is not entirely surprising since it seems likely that in the medulla tyrosine is converted to dihydroxyphenylalanine. This involves the substitution of a hydroxyl group in a benzene ring.

c) The function of citric acid cycle intermediates in DOC 11β-hydroxylation.

Any explanation of steroid 11β-hydroxylation must account for the role of members of the citric acid cycle in the reaction. The results of the present investigation have confirmed the observations of other workers that the presence of these citric acid cycle intermediates permits the steroid 11β-hydroxylation to proceed. It has been shown that the efficiency of these intermediates as 'activators' of DOC 11β-hydroxylation depends largely on the

ability of the enzyme preparation used to oxidize them. It has been shown that DOC 11β-hydroxylation is inhibited by malonate in the presence of succinate. Succinate oxidation is inhibited in this case by malonate inhibition of succinic dehydrogenase. The 'single step' oxidation of α-oxoglutarate to succinate obtained in the malonate blocked system has been shown to permit DOC 11β-hydroxylation. This observation apparently eliminates the possibility that fumarate has some specific role in the 11β-hydroxylation reaction in intact mitochondria. The failure of fumarate to stimulate 11β-hydroxylation under anaerobic conditions and the failure of high concentrations of succinate to inhibit 11β-hydroxylation do not support the suggestion that fumarate acts as a hydrogen acceptor. It seems most likely that fumarate enters the citric acid cycle and thereby provides substrates for oxidative reactions coupled with DOC 11β-hydroxylation.

Although all citric acid cycle intermediates tried did permit DOC 11β-hydroxylation to occur in ox-adrenocortical mitochondria, it was observed that the amount of hydroxylation varied with each intermediate used. Some explanation of this can be offered. In general, the amount of 11β-hydroxylation occurring depended on the extent of oxidation of the

oxidizable substrate. Oxaloacetate is not an efficient activator of 11β-hydroxylation. Low concentrations of this substance inhibit oxidation of succinate (Das, 1937) and malate (Elliot, 1941). The poor activation obtained with citrate is not explained by the ability of this substance to remove magnesium ions. The observation that citrate does not readily pass through mitochondrial membranes (Schneider, 1953) offers one explanation of this poor activation of DOC 11β-hydroxylation. In addition, it is known that the isocitric dehydrogenase activity of isolated mitochondria is low (Hogeboom & Schneider, 1950). In cell fractionation studies they found most of the isocitric dehydrogenase activity associated with the soluble protein fraction obtained on high speed centrifugation of homogenates. These observations indicate a limitation of the in vitro technique. It is most probable that in the intact gland no such differences between various intermediates would be found. These differences are most likely due to alterations in the activity of the mitochondria occurring in the isolation procedure used. The activity of the mitochondria does depend on the other components of the cell. In addition, in the intact gland the mitochondria are not normally confronted with the necessity to utilize high

concentrations of individual members of the citric acid cycle. Normally optimal concentrations of citric acid cycle intermediates are being metabolized and, presumably, oxidation of these can be coupled in some way with the 11β-hydroxylation reaction.

Kahnt & Wettstein (1951) suggested that oxidative phosphorylation might be involved in steroid 11β-hydroxylation, but hitherto no evidence has been produced to support this suggestion. It has now been shown that 2:4-dinitrophenol inhibits the 11β-hydroxylation of DOC without decreasing the oxidation of the activator. 2:4-Dinitrophenol, used in low concentrations, is well known to uncouple phosphorylation from oxidation (Loomis & Lipmann, 1948). One role of members of the citric acid cycle in the 11β-hydroxylation of DOC would thus appear to be that of oxidizable substrates for processes of oxidative phosphorylation involved, perhaps, in the activation of the DOC before hydroxylation. It is possible that ATP, generated by the oxidation of citric acid cycle intermediates, is involved in activating the steroid prior to 11β-hydroxylation. This may simply be the formation of a water-soluble intermediate, thus increasing the rate of reaction with the hydroxylating enzyme system.

In view of the successful linking of DOC 11β-hydroxylation with the 'single step' oxidation of α-oxoglutarate to succinate, it is interesting to note that Copenhaver & Lardy (1952) observed the highest 'phosphorus esterified/oxygen consumed' ratio for this particular step of the citric acid cycle.

Dorfman (1953) reported that, using the washed residue type of preparation (Hayano & Dorfman, 1953), no increase in oxygen uptake was observed in the presence of fumarate as against no fumarate. This is a very surprising observation considering that these preparations do contain mitochondria. It must be assumed that the integrity of the mitochondria is so altered by the method of preparation of the residue that they can no longer catalyze the normal reactions of the citric acid cycle. Under these circumstances the above authors have claimed the specificity of fumarate as an activator of DOC 11β-hydroxylation. It is probable that such an observation is due to the damaged nature of the enzyme preparation used.

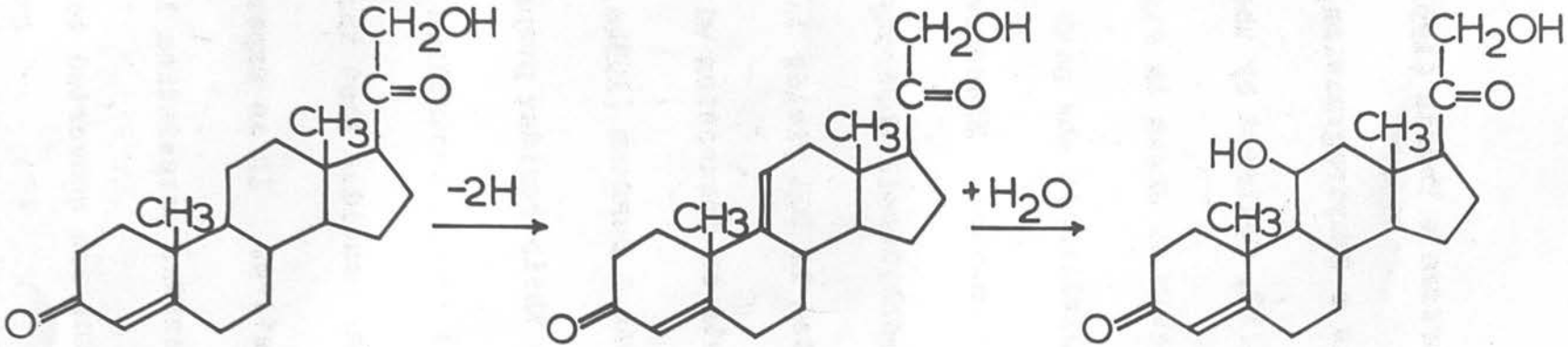
Only those oxidations involving members of the citric acid cycle as substrates have been considered in this report. It is possible, however, that other oxidations coupled with phosphorylation

occurring in adrenocortical cells, are equally capable of 'activating' DOC 11β-hydroxylation. Since it is probable that in many cases oxidative phosphorylation is associated with hydrogen- and electron-transporting steps of biological oxidation, rather than with primary dehydrogenation (Hunter, 1951), oxidation of reduced pyridine nucleotide coenzymes would be expected to activate steroid 11β-hydroxylation. In this case, it would be necessary to distinguish between cofactors involved in energy-producing reactions, and those concerned more directly with steroid hydroxylations. In this connection the observation of Hayano, Wiener & Lindberg (1953) is of interest. They found that triphosphopyridine nucleotide alone was able to replace the three cofactors-magnesium ions, ATP and diphosphopyridine nucleotide - which were required to regenerate the steroid-hydroxylating activity of enzyme preparations from stored adrenals. A probable interpretation of this work is that TPN, required for some step of the hydroxylation reaction, is removed from the stored adrenal cells on washing. The enzyme preparation may still retain the ability to synthesize TPN from DPN and ATP with magnesium ions as cofactor by reactions described by Kornberg (1951). More recently Hayano & Dorfman

(1954a) have shown that in extracts of acetone powders prepared from adrenal homogenate residues (Hayano & Dorfman, 1953) the 11β-hydroxylating activity can be restored by the addition of fumarate, TPN and oxygen. 2:4-Dinitrophenol does not inhibit 11β-hydroxylation in this system, which would suggest that coupled oxidative phosphorylation is required for the production of TPN from DPN. In this laboratory, Dr Grant and the present author have recently shown, using a soluble enzyme preparation of acetone powder prepared from adrenocortical mitochondria, that TPN and fumarate are required for DOC 11β-hydroxylation. It remains to be proved that TPN acts as a hydrogen acceptor for a reaction in which DOC is directly involved.

The observation that added ATP alone is unable to activate DOC 11β-hydroxylation may be related to the similar observation of Kennedy & Lehninger (1948, 1951) in the case of the 'priming' of fatty acid oxidation. This may find an explanation in the fact that added ATP is less readily available for intra-mitochondrial reactions than that generated within the mitochondria (Siekevitz & Potter, 1953).

Proposed scheme for 11β-hydroxylation.



11-deoxycorticosterone. 9,11-anhydrocorticosterone. corticosterone.

Fig.16

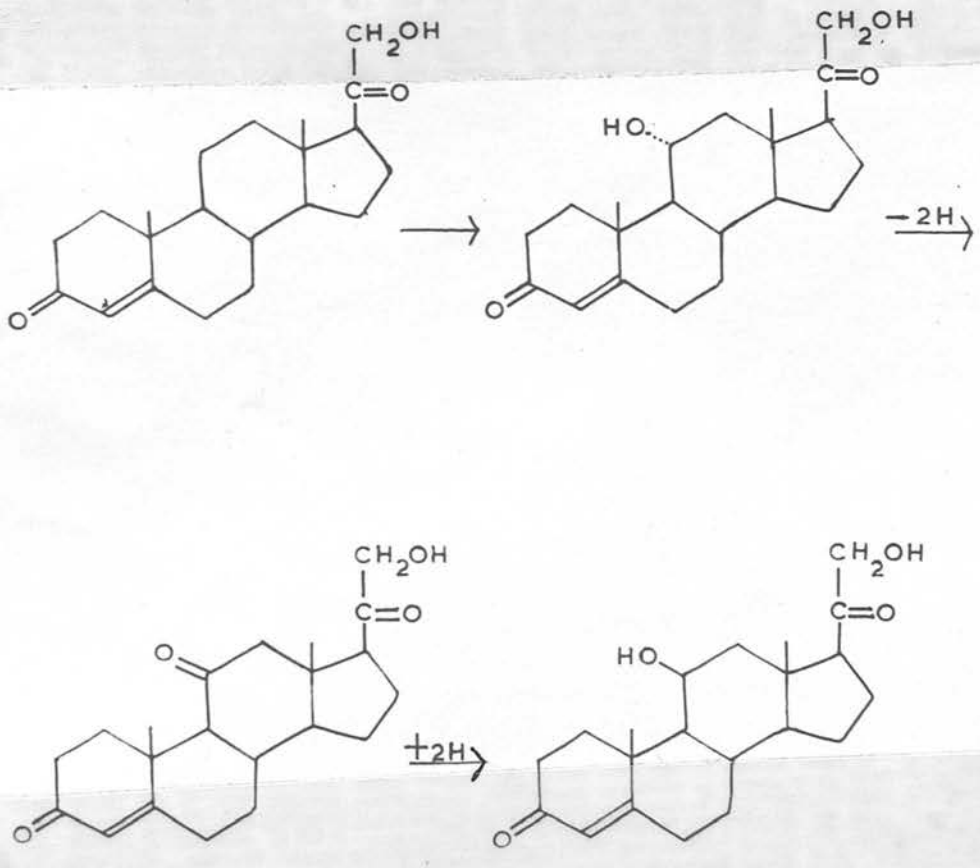
d) The mechanism of steroid 11β-hydroxylation

With regard to the direct mechanism involved in steroid 11β-hydroxylation, four mechanisms have been suggested.

i) Hayano, Dorfman & Yamada (1951) suggested that first there is a dehydrogenation, either at carbons 9:11 or 11:12, followed by the addition of a molecule of water as shown in Fig. 16. This mechanism is similar to the path from succinate, through fumarate, to malate. Miescher et al. (1953) reported that 9:11-anhydro-17α-hydroxy-corticosterone acetate was converted in 20% yields to 11-hydroxylated products on incubation with adrenal homogenates. Hayano & Dorfman (1954a) have repeated this using their residue preparation and have obtained only 5 to 10% production of a more polar product. They consider that these results are essentially negative. In an experiment with D_2O using adrenal residue preparation, 17α-hydroxy-11-deoxycorticosterone was converted to 17α-hydroxycorticosterone but no deuterium entered a stable position in the molecule. They suggest that these facts exclude the possibility of any biosynthetic mechanism involving the direct addition of water. The possibility of an 9:11 or 11:12 unsaturated steroid being involved as an intermediary in 11β-hydroxylation is unlikely. While the

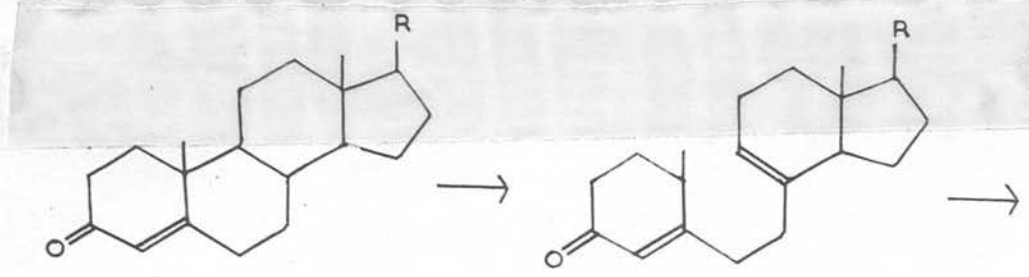
latter idea was still prevalent, fumarate and/or TPN were thought to be involved in the production of the unsaturated steroid intermediate. However, it has been shown that with intact mitochondria, fumarate is probably not a necessity for DOC 11β-hydroxylation. It seems likely that in the intact gland fumarate has no special function, and that it is only in damaged enzyme preparations that it becomes necessary for steroid 11β-hydroxylation. In such damaged preparations (adrenal residues and acetone powders) TPN is essential for 11β-hydroxylation, but it has not been shown whether in the intact gland there is any such requirement. If an unsaturated steroid intermediate is not involved in steroid 11β-hydroxylation then some other role of fumarate and TPN must be sought.

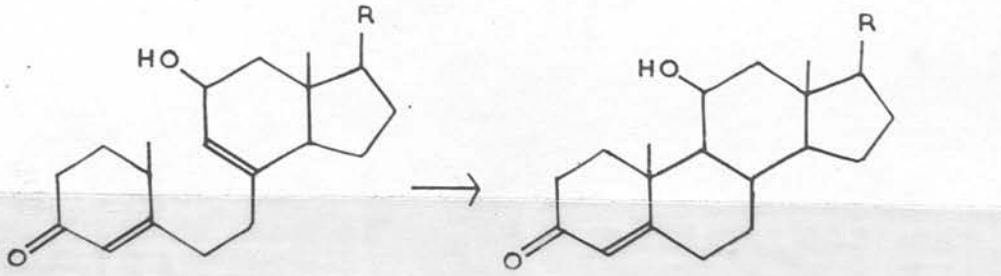
ii) From the point of view of steric considerations, 11β-hydroxylation is an interesting reaction. The hydrogen at 11β- is chemically inactive, whereas the 11α-hydrogen is reactive. To account for the conversion by 11β-hydroxylation of DOC to corticosterone, the following reaction sequence has been suggested.



In this sequence there is first an attack on the reactive 11α-hydrogen to form 11α-hydroxy DOC, followed by dehydrogenation to the 11 ketone, then by reduction to the 11β-hydroxy DOC (corticosterone). It is possible that TPN and fumarate play some role in these oxidations and reductions. However, perfusion of cortisone through whole adrenals (Meyer, 1953),or incubation with adrenal homogenate (Hayano & Dorfman, 1954b) leads to production of the 11β-hydroxy derivative in only trace amounts.

iii) Fieser (1953) has suggested that 11β-hydroxylation may follow the sequence of formation of a seco derivative, allylic oxidation at C-11, and ring closure, as shown below. There is no evidence for or against this view as yet.





iv) Levy et al. (1953) have suggested a mechanism of 11β-hydroxylation whereby hydroxyl free radicals are involved in the direct oxidation of DOC. One of the free radicals would come in and take off the α-hydrogen at the 11-position, and another would replace the hydrogen with subsequent inversion of configuration. They suggest that dicarboxylic acids

of the citric acid cycle on being oxidized, involving the hydrogen and electron-transporting mechanism, might result in the production of a peroxide. The peroxide formed might then be the precursor of the hydroxyl free radical.

It can be seen that no clear ideas have been obtained so far on the mechanisms of the 11β-hydroxylation reaction. It seems that the most profitable approach to this problem may be via spectrophotometric investigations using a soluble 11β-hydroxylation enzyme system. (In collaboration with Dr Grant, such investigations are being carried out.)

2) 11β-Hydroxylation of progesterone by ox-adrenocortical mitochondria.

Hechter (1953a) cautiously describes the scheme of adrenocortical steroid biogenesis (Fig. 11) which he previously advanced (Hechter et al., 1951) as a 'series of deductions based primarily upon the ability of substances to react', and admits the possibility that such in vitro reactions may be relatively unimportant in normal metabolic pathways. When evaluating the physiological significance of

in vitro reactions of adrenocortical steroids and their possible precursors, it is important to bear certain points in mind. The state of the enzymes employed and the influence of the steroids on enzyme reactions are important factors which merit particular attention. In the discussion on DOC 11β-hydroxylation, the unsatisfactory nature of homogenate and washed sediments used as sources of enzymes for the study of adrenocortical steroid metabolism was pointed out. Even in the perfusion technique of Hechter *et al.* (1953), steroids are exposed to deliberately damaged adrenal tissue.

Little attention has been paid to the influence of steroids on reactions involved in the biogenesis of adrenocortical steroids. Hechter (1953b) has referred to the possible influence of sex hormones on the pattern of adrenocortical steroids synthesized and released from the gland.

Sourkes & Heneage (1952) have observed a stimulation by DOC of succinate oxidation by rat adrenal halves. Brummel, Halkerston & Reiss (1954) have made the same observation with ox-adrenal slices. This stimulation of succinate oxidation has now been observed with ox-adrenocortical mitochondria. At the same time, the DOC is extensively converted to corticosterone. The oxygen required for this reaction

does not entirely account for the increased oxygen-consumption observed. In contrast with the effect of DOC, it has now been found that progesterone inhibits succinate oxidation by ox-adrenocortical mitochondria and that under these conditions, the 11β-hydroxylation of the progesterone is limited. Progesterone also has a greater inhibitory effect than DOC on the reactions involved in the synthesis of citrate from pyruvate and L-malate. The inhibition by progesterone of succinate oxidation is most marked during the first 10 minutes of incubation. Thereafter, oxygen uptake is almost parallel with that in control experiments without added steroid (Fig. 12). It appears that the enzymes directly or indirectly concerned in the 11β-hydroxylation of steroids by ox-adrenocortical mitochondria suffer permanent damage during the first 10 minutes of incubation if citric acid cycle acids are absent, or if their oxidation is inhibited by the presence of progesterone (p. 86). This may be explained by the well-known instability under these conditions of enzymes involved in oxidative phosphorylation. It has previously been shown that oxidative phosphorylation is required for 11β-hydroxylation to occur in intact mitochondria.

By increasing the enzyme and succinate concentrations it was possible to overcome the initial

inhibition by progesterone of oxygen uptake. Under these conditions (p. 89) appreciable metabolism of the progesterone occurred. This metabolism of progesterone was shown to be mainly 11β-hydroxylation to give 11β-hydroxyprogesterone. Again it has been found that efficient 11β-hydroxylation of steroids will only occur in intact mitochondria in the presence of some citric acid cycle intermediate being efficiently oxidized. Progesterone itself tends to inhibit such oxidations, and thus affects the extent of its own 11β-hydroxylation by adrenocortical mitochondria. The increased rate of ATP breakdown by rat tissues in the presence of progesterone observed by Jones & Wade (1953) offers an additional explanation for the influence of progesterone on reactions concerned with 11β-hydroxylation.

In the scheme of adrenocortical steroid biogenesis advanced by Hechter et al. (1951), 11β-hydroxy-progesterone is shown as a possible intermediate in the formation of corticosterone or 17α-hydroxy-corticosterone from progesterone. The status of 11β-hydroxyprogesterone as such an intermediary is diminished by the following facts. This steroid has hitherto only been isolated in yields of less than 1% after perfusion through adrenal glands. It has not been found among the products on incubation of

progesterone with adrenocortical tissue preparations (Hayano & Dorfman, 1952, 1953). Finally, 11β-hydroxyprogesterone has not been converted into corticosterone or 17α-hydroxycorticosterone on perfusion through adrenals (Jeanloz et al. 1954). Since it has now been possible to convert progesterone to 11β-hydroxyprogesterone in good yield by ox-adrenocortical mitochondria, it would appear that the status of the latter steroid as an intermediary in the biosynthesis of adrenocortical steroids should be reconsidered. The conditions of the perfusion experiments of Jeanloz et al. have not been reported so it is not possible to decide finally whether further metabolism of 11β-hydroxyprogesterone does occur.

It is not as yet possible to ascertain the extent to which progesterone is converted to 11β-hydroxy-progesterone in the intact animal - if at all. However, it has been recently shown by Saba, Hechter & Stone (1954) that the primary degradation of cholesterol appears to take place in the mitochondrial fraction and, if this gives rise to progesterone, this steroid may be converted to 11β-hydroxyprogesterone in the mitochondria before passing into the cytoplasm to be 17- and 21-hydroxylated.

The elucidation of the mechanism of adrenocortical-steroid biogenesis is further limited by lack of knowledge of the influence of other steroids on the metabolism of their precursors. It has been shown that in ox-adrenocortical mitochondria, 11β-hydroxylation of DOC is inhibited in the presence of progesterone. In what way this may apply to the gland *in vivo* cannot be ascertained at present. These observations have, however, indicated the importance of considering the effect of these steroids on reactions involved in their own synthesis and metabolism. There is some evidence that ACTH increases adrenocortical steroid production by increasing the rate of some reaction at a stage prior to the probable production of progesterone from cholesterol or some similar precursor. If this leads to an increased production of progesterone it is interesting to speculate how this will affect 11β-hydroxylation of steroids in the gland. One possible effect may be to inhibit the 11β-hydroxylation reactions by inhibiting reactions supporting 11β-hydroxylation and in this way the progesterone itself would not be 11β-hydroxylated. There is considerable evidence that after 11β-hydroxylation no further hydroxylation of a steroid can occur in the adrenal. If this is so, and if the increased

progesterone production prevents normal 11β-hydroxylation occurring, then presumably the progesterone can be first 17- and 21-hydroxylated by the soluble enzymes of the cytoplasm obtained in the supernatant fraction of a centrifuged adrenal homogenate. 11β-Hydroxylation of these hydroxylated derivatives could then occur in the mitochondria; progesterone inhibition of 11β-hydroxylation having been removed by conversion to these 17- and 21-hydroxylated compounds.

It can be seen that the status of progesterone metabolism in the adrenal is not completely clear. It is the present author's contention that this problem will only be satisfactorily settled when due consideration is taken of the effect of progesterone on metabolic reactions in general within the adrenal gland.

I

REFERENCES

Atherden, L.M. (1954). Personal communication.

Axelrod, L.R. & Miller, L.L. (1953). Fed. Proc. 12, 173.

Bernstein, S. & Leuhard, R.H. (1953). J. Org. Chem. 18, 1146.

Berthet, J., Berthet, L., Appelmans, F. & Duve, C. de (1951). Biochem. J. 50, 182.

Brendler, H. (1951). Science, 114, 61.

Brockmann, H. & Schodder, H. (1941). Ber. dtsch. chem. Ges. 74, 73.

Brummel, E., Halkerston, I.D.K. & Reiss, M. (1954). J. Endocrin. 10, 111.

Bush, I.E. (1952). Biochem. J. 50, 370.

Butt, W.R., Morris, P., Morris, C.J.O.R. & Williams, D.C. (1951). Biochem. J. 49, 434.

Cohen, S. (1953). J. biol. Chem. 201, 93.

Copenhaver, J.H. & Lardy, H.A. (1952). J. biol. Chem. 195, 225.

Craig, L.C. & Craig, D. (1950). Techniques of Organic Chemistry, vol. 3, ed. Weissberger. New York: Interscience Publishers.

Daughaday, W.H., Jaffe, H. & Williams, R.H. (1948). J. clin. Endocrin. 8, 166.

Dorfman, R.I., Hayano, M., Haynes, R. & Savard, K. (1953). In Ciba Foundation colloquia on Endocrinology, vol. 7, p. 202.

Das, N.B. (1937). Biochem. J. 31, 1124.

Elliot, K.A.C. (1941). Physiol. Rev. 21, 267.

Fieser, L.F. (1953). In Ciba Colloquia on Endocrinology, vol. 7, 288.

Grant, J.K. & Taylor, W. (1952). Biochem. J. 52, xxiv.

Green, D.E., Loomis, W.F. & Auerbach, V.H. (1948). J. biol. Chem. 172, 389.

Haines, W.J. (1952). Recent Progr. Hormone Res. 7, 255.

Harman, J.W. (1950). Exp. Cell Res. 1, 382.

Hayano, M. & Dorfman, R.I. (1952). Arch. Biochem. Biophys. 36, 237.

Hayano, M. & Dorfman, R.I. (1953). J. biol. Chem. 201, 175.

Hayano, M. & Dorfman, R.I. (1954a). J. biol. Chem. 211, 227.

Hayano, M. & Dorfman, R.I. (1954b). Unpublished observations.

Hayano, M., Dorfman, R.I. & Prins, D.A. (1949). Proc. Soc. exp. Biol., N.Y., 72, 700.

Hayano, M., Dorfman, R.I. & Yamada, E.Y. (1951). J. biol. Chem. 193, 175.

Hayano, M., Wiener, M. & Lindberg, M.C. (1953). Fed. Proc. 12, 216.

Hechter, O. (1953a). In Ciba Foundation Colloquia on Endocrinology, vol. 7, p. 161.

Hechter, O. (1953b). Rev. Canad. Biol. 12, 123.

Hechter, O., Jacobsen, R.P., Jeanloz, R.W., Levy, H., Marshall, C.W., Pincus, G. & Schenker, V. (1949). J. Amer. Chem. Soc. 71, 3261.

Hechter, O., Jacobsen, R.P., Jeanloz, R.W., Levy, H., Marshall, C.W., Pincus, G. & Schenker, V. (1950). Arch. Biochem. 25, 457.

Hechter, O., Jacobsen, R.P., Schenker, V., Levy, H., Jeanloz, R.W., Marshall, C.W. & Pincus, G. (1953). Endocrinology, 52, 679.

Hechter, O., Zaffaroni, A., Jacobsen, R.P., Levy, H., Jeanloz, R.W., Schenker, V. & Pincus, G. (1951). Recent Progr. Hormone Res. 6, 215.

Heunnekens, F.M. & Green, D.E. (1950). Arch. Biochem. 27, 418.

Hogeboom, G.H. & Schneider, W.C. (1950). J. biol. Chem. 186, 417.

Hubener, H.J. & Amelung, D. (1953). Z. physiol. Chem. 293, 126.

Hubener, H.J. & Schmidt-Thome, J. (1955). Z. physiol. Chem. 299, 240.

Hunter, F.E. (1951). In Phosphorus Metabolism, 1, 297. eds. W.D. McElroy & B. Glass, Baltimore. The Johns Hopkins Press.

Jeanloz, R.W., Levy, H., Jacobsen, R.P., Hechter, O., Schenker, V. & Pincus, G. (1954). Unpublished observations.

Jones, H.W. jun. & Wade, R. (1953). Science, 118, 103.

Kahnt, F.W. (1953). In Ciba Foundation Colloquia on Endocrinology, vol. 7, p. 203.

Kahnt, F.W. & Wettstein, A. (1951). Helv. chim. acta. 34, 1790.

Kennedy, E.P. & Lehninger, A.L. (1949). J. biol. Chem. 179, 957.

Kennedy, E.P. & Lehninger, A.L. (1951). J. biol. Chem. 190, 361.

Klyne, W. & Patterson, J.Y.F. (1948). Biochem. J. 42, 1.

Klyne, W. & Rankeillor, J. (1947). Chem. & Ind. 17, 224.

Kochakian, C.D. (1951). Ann. New York Acad. Sci. 54, 534.

Kornberg, A. (1951). In Phosphorus Metabolism, 1, 392. eds. W.A. McElroy & B. Glass, Baltimore. The Johns Hopkins Press.

Lehninger, A.L. & Kennedy, E.P. (1948). J. biol. Chem. 173, 753.

Levy, H., Jeanloz, R.W., Marshall, C.W., Jacobsen, R.P., Hechter, O., Schenker, V. & Pincus, G. (1953). J. biol. Chem. 203, 433.

Loomis, W.F. & Lipmann, F. (1948). J. biol. Chem. 173, 807.

McGinty, D.A., Smith, G.N., Wilson, M.L. & Worrel, C.S. (1950). Science, 112, 506.

Mader, W.J. & Buck, R.R. (1952). Analyt. Chem. 24, 666.

Mann, P.J.G. & Quastel, J.H. (1941). Biochem. J. 35, 502.

Mattox, V.R. & Kendall, E.C. (1951). J. biol. Chem. 188, 287.

Meyer, A. (1953). J. biol. Chem. 203, 469.

Miescher, K., Wettstein, A. & Kahnt, F.W. (1953). Acta physiol. Latinoamericana, 3, 144.

Morris, C.J.O.R. (1953). Personal communication.

Neher, R. & Wettstein, A. (1951). Helv. chim. acta, 34, 2278.

Plager, J.E. & Samuels, L.T. (1953). Arch. Biochem. Biophys. 42, 477.

Potter, V.R. & Elvehjem, C.A. (1936). J. biol. Chem. 114, 495.

Potter, V.R., Lyle, G.G. & Schneider, W.C. (1951). J. biol. Chem. 190, 293.

Potter, V.R., Recknagel, R.O. & Hurlbert, R.B. (1951). Fed. Proc. 10, 646.

Reichstein, T. & Euw, J. von (1938). Helv. chim. acta, 21, 1197.

Reichstein, T. & Fuchs, H.G. (1940). Helv. chim. acta, 23, 684.

Saba, N., Hechter, O. & Stone, D. (1954). J. Amer. chem. Soc. 76, 3862.

Sarkar, N.K., Beinert, H., Fuld, M. & Green, D.E. (1952). Arch. Biochem. Biophys. 37, 140.

Savard, K., Green, A.A.& Lewis, L.A. (1950). Endocrinology, 47, 418.

Schneider, W.C. (1949). In Manometric Techniques and Tissue Metabolism, p. 148, by W.W. Umbreit. R.H. Burris & J.F. Stauffer, Minneapolis: Burgess Publishing Co.

Schneider, J.J. (1952). J. biol. Chem. 199, 235.

Schneider, W.C. (1953). J. Histochem. Cytochem. 1, 212.

Schneider, W.C. & Hogeboom, G.H. (1951). Cancer Res. 11, 1.

Schwarz, V. (1952). Biochem. J. 52, xiii.

Seneca, H., Ellenbogen, E., Henderson, E., Collins, A. & Rockenbach, J. (1950). Science, 112, 524.

Siekevitz, P. & Potter, V.R. (1953). J. biol. Chem. 201, 1.

Sourkes, T.L. & Heneage, P. (1952). Endocrinology, 50, 73.

Steiger, M. & Reichstein, T. (1937). Helv. chim. acta, 20, 1164.

Sweat, M.L. (1951). J. Amer. chem. Soc. 73, 4056.

Taylor, T.G. (1953). Biochem. J. 54, 48.

Taylor, W. (1953). Thesis presented for the Degree of Doctor of Philosophy, University of Edinburgh.

Taylor, W. (1954). Biochem. J. 56, 463.

Umbreit, W.W. (1949). In Manometric Techniques and Tissue Metabolism, p. 1, by W.W. Umbreit. R.H. Burris & J.F. Stauffer. Minneapolis: Burgess Publishing Co.

Witter, R.F., Pories, W.J. & Cottons, M.A. (1953). Proc. Soc. exp. Biol. Med. 84, 674.

Zaffaroni, A. (1950). J. Amer. chem. Soc. 72, 3828.

[From THE BIOCHEMICAL JOURNAL, Vol. 57, No. 2, pp. 255–263, 1954]

PRINTED IN GREAT BRITAIN

The *in vitro* Enzymic Hydroxylation of Steroid Hormones

1. FACTORS INFLUENCING THE ENZYMIC 11β-HYDROXYLATION OF 11-DEOXYCORTICOSTERONE

By A. C. BROWNIE and J. K. GRANT
Biochemistry Department, University of Edinburgh

(*Received* 13 *October* 1953)

The appreciation of the profound influence of steroid hormones of the adrenal gland on metabolism, and an interest in the biogenesis of adrenocortical hormones stimulated many workers to investigate enzymic hydroxylation of adrenal steroids. Thus Hayano, Dorfman & Prins (1949) and Hayano, Dorfman & Yamada (1951) incubated 11-deoxycorticosterone (DOC) with adrenal slices, minces and 'homogenates' and observed an increased glycogenic activity in the products which they ascribed to the formation of 11β-hydroxysteroids. Adenosine triphosphate (ATP), fumarate and magnesium ions stimulated the reaction when 'homogenates' were used. Savard, Green & Lewis (1950) obtained evidence for the formation of 11β-hydroxy derivatives from DOC and 17α-hydroxy-11-deoxycorticosterone on incubation with adrenal 'homogenates' without added fumarate or other cofactors. McGinty, Smith, Wilson & Worrel (1950) isolated 17α-hydroxycorticosterone as a transformation product of 17α-hydroxy-11-deoxycorticosterone incubated with adrenal 'homogenates' supplemented with glucose and fumarate. Kahnt & Wettstein (1951) found that ATP or adenylic acid could replace fumarate as a cofactor in the 11β-hydroxylation reaction. Nicotinamide stimulated the reaction, suggesting a requirement for pyridine nucleotides. Glucose, inorganic phosphate and magnesium ions were without effect. These authors suggested that processes of oxidative phosphorylation may be involved, and that the steroid hydroxylation may be catalysed by enzymes of Green's 'cyclophorase' system (Green, Loomis & Auerbach, 1948). 'Cyclophorase' preparations, however, are not cytoplasmically homogeneous (Harman, 1950). Credit for showing that 11β-hydroxylating enzymes are associated with adrenal-cell particles which by their method of preparation were mainly

mitochondria, is due to Sweat (1951). He isolated crystalline 17α-hydroxycorticosterone after incubation of 17α-hydroxy-11-deoxycorticosterone with the adrenal-cell particles in a medium supplemented with glucose, fumarate and magnesium ions. Addition of ATP was not necessary. Recently, Hayano & Dorfman (1953) have extended their studies of steroid hydroxylation using 'residue preparations of twice washed ox-adrenal "homogenates" obtained at 5000 *g*'. DOC was converted predominantly into corticosterone but other unidentified products were also detected. The authors claim to have demonstrated the 'specificity and absolute necessity for fumarate and magnesium ions and the stimulating capacity of ATP and diphosphopyridine nucleotide (DPN)'. They suggest that fumarate may play some part in an energy-yielding system for the regeneration of ATP, or that it may function as a hydrogen acceptor in the steroid-hydroxylation reaction.

Much of the previous work on enzymic hydroxylation of steroids is open to the criticisms that enzyme preparations and methods of steroid analysis have not been satisfactory. These criticisms will be dealt with fully in the Discussion. Probably on account of these features of earlier investigations, the cofactor requirement for the enzymic *in vitro* hydroxylation of steroids is at present unsettled. The role of cofactors thought to be necessary is unknown and the mechanism of the 11β-hydroxylation reaction remains to be elucidated. As regards the mechanism of hydroxylation, Hayano & Dorfman (1953) have suggested the formation of an unsaturated intermediate which adds on the elements of water, whereas Levy *et al.* (1953) propose a simpler mechanism involving formation of a steroid free radical. As the authors admit, these views are at present no more than speculation.

With a view to studying these outstanding problems a preliminary investigation of the *in vitro* enzymic 11β-hydroxylation of DOC has been made and is now reported. For this purpose a purified mitochondrial preparation from ox-adrenal cortical tissue has been used. Advantage has been taken of a specific, sensitive and accurate method for the determination of DOC and related steroids in tissue preparations which has been developed in this Department (Taylor, 1954).

A preliminary report of this work has been communicated to the Biochemical Society (Brownie, Grant & Taylor, 1953).

EXPERIMENTAL

Preparation of mitochondria

Adrenal glands were obtained from the slaughterhouse from oxen killed by stunning and exsanguination. The glands were removed from the carcasses within 20 min. of death, stripped of fat and connective tissue and placed in a deep-freeze cabinet at −20° until collected for transport to the laboratory packed in crushed ice. Subsequent operations were performed at 0–4° and completed within 3 hr. of the death of the animal. Cortical tissues (15 g.) were separated and finely ground with 45 ml. isotonic (0·25 M) sucrose using a loosely fitting pestle of an all-glass 'homogenizer' (Potter & Elvehjem, 1936). Grinding of the tissue was continued in an apparatus of similar design having a glass tube of accurately uniform internal diameter and a well-fitting Nylon pestle grooved longitudinally at three points. This apparatus has the advantage of producing 'homogenates' with few intact cells. It shows no signs of wear in use over long periods (cf. Brendler, 1951). The two-stage preparation of the 'homogenate' was adopted since it was found that adrenocortical cells were not readily broken by procedures required for the preliminary disruption of the tissue. The Waring Blendor type of apparatus was not used since it was found to give a product of low enzymic activity when run at high speed, and unacceptably low yields of mitochondria when run at low speeds. The former observation is in agreement with the findings of Stern & Bird (1949) and of Lambden (1950).

The final 'homogenate' was diluted to 90 ml. with 0·25 M sucrose. Nuclei and intact cells were removed by centrifuging for 10 min. at 700 *g*. The supernatant was carefully removed and centrifuged twice at 5000 *g* for 10 min. to sediment mitochondria. Care was taken to obtain as pure a mitochondrial preparation as possible rather than a quantitative yield of these particles. Thus poorly packed mitochondria which might contain some microsomes were discarded during removal of the supernatant (cf. Schneider, 1949). The mitochondrial pellets were combined, washed with 0·25 M sucrose and resedimented at 21 000 *g* for 10 min. In later preparations it has been found convenient to obtain firmly packed mitochondria by final sedimentation from isotonic (0·154 M) KCl. The wet weight of the mitochondrial pellet (about 2 g.) was determined after decanting the supernatant fluid and carefully drying the walls of the centrifuge tube with filter paper. The mitochondria were finally thoroughly dispersed in 9 vol. 0·154 M-KCl. Preparations from the last sucrose wash were occasionally examined under a phase-contrast microscope. Whole cells were never observed, single nuclei very occasionally. Purity was also occasionally checked by a final centrifugation of part of the preparation from a 0·01 % solution of janus green B prepared in 0·25 M sucrose. On incubation for 5 min. at 37° the blue-stained pellet in the centrifuge tube turned red. This reaction is claimed to be specific for mitochondria (Potter, Rechnagel & Hurlbert, 1951). The pellet is not disturbed in this test. If nuclei and microsomes are present as contaminants they may be distinguished at the lower and upper surfaces of the pellet by their staining characteristics which differ from those of the mitochondria.

Preparation of 11-*deoxycorticosterone*

11-Deoxycorticosterone acetate was hydrolysed at 37° by the method of Mattox & Kendall (1951). DOC was recovered from the acid hydrolysate by extraction with $CHCl_3$. The residue obtained after distillation of the washed $CHCl_3$ extract was crystallized from ether and ether:acetone (Reichstein & Euw, 1938) to give a product melting at 138–142°, unchanged by admixture with an authentic sample of DOC. $[\alpha]_D^{25}$ +178·8±1·2° in ethanol (*c*, 0·493).

Table 1. *Recovery of DOC and corticosterone added to reaction mixtures which had been incubated with adrenocortical mitochondria*

Values have been corrected by subtraction of 'apparent' steroid recovered in blank control experiments in which no steroids were added—20μg. for either steroid.

Extraction control experiments					
DOC			Corticosterone		
Added (μg.)	Recovered (μg.)	Recovery (%)	Added (μg.)	Recovered (μg.)	Recovery (%)
524	500	96	125	122	97
470	465	99	99	97	98
36	34	94	36	33	92

Steiger & Reichstein (1937) reported $[\alpha]_D^{20} + 178 \pm 3°$ for DOC in ethanol (*c*, 1·5). (Found: C, 76·3; H, 9·2. Calc. for $C_{21}H_{30}O_3$: C, 76·2; H, 9·1%.)

Incubation conditions

Unless otherwise stated, in all experiments 25 ml. conical flasks were charged with the following reaction mixture: 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·04 M potassium phosphate, pH 7·4, and a mitochondrial suspension in KCl solution containing about 1·4 mg. total N. The total vol. was 3 ml. Members of the citric acid cycle, cofactors or inhibitors were added as potassium salts wherever possible in solution at pH 7·4. When additions were made to the reaction mixture the amount of KCl present was adjusted to maintain a total cationic concentration of 0·141–0·151 M. A propylene glycol (propane-1:2-diol) solution of DOC, containing about 500 μg. steroid in 0·04 ml., was added from a Burroughs Wellcome 'Agla' microburette immediately after addition of the mitochondrial preparation. The best results were obtained in this way and it seems likely that the steroid is precipitated out of solution on to the mitochondrial particles and is thus more readily assimilated. Glass-distilled water was used for all solutions and pH values were checked by glass electrode. Unless otherwise stated, incubations were in air, shaking for 1 hr. at 37°.

Determination of DOC and corticosterone

DOC and corticosterone in reaction mixtures were determined by an adaptation of the method described by Taylor (1954) for progesterone. Acetone extracts were not chilled. The aqueous-acetone filtrate obtained after removal of protein was distilled to an aqueous residue from which steroids were extracted by benzene:chloroform (6:1,v/v). DOC in a portion of this extract was separated by partition chromatography on a Celite column using a benzene:hexane (3:7, v/v) mobile phase and methanol:water (7:3, v/v) stationary phase. The eluate fraction from 15 to 25 ml. contained DOC. Corticosterone was separated similarly from another portion of the benzene:chloroform extract using the solvent system benzene:hexane (8:2, v/v), methanol:water (7:3, v/v), and was eluted in the fraction from 18 to 26 ml. Fractions containing DOC or corticosterone were evaporated to dryness under an air stream. The residues were taken up in ethanol and the absorption of solutions measured at 240 mμ. in a 1 cm. cell of a Unicam SP 500 Spectrophotometer. The amounts of steroid present were found by reference to calibration curves.

Typical results for the recovery of DOC and corticosterone added to incubated reaction mixtures are shown in Table 1.

Table 2. *Recovery of steroids absorbing selectively at 240 mμ. in DOC and corticosterone chromatogram fractions after incubation of DOC with adrenocortical mitochondria*

DOC incubated (μmoles)	DOC recovered (μmoles)	Corticosterone recovered (μmoles)	Total recovery (μmoles)	Total recovery (%)
1·72	0·26	1·32	1·58	92
1·42	0·27	1·07	1·34	94·5

In a series of 20 experiments DOC recoveries at the 500 μg. level were 96 $\pm$ 1%.

Every experiment included duplicate flasks to which DOC was added after incubation to permit a check to be made of the extraction procedure ('extraction controls'). 'Apparent' DOC or corticosterone was determined in experiments without added steroid ('blank controls'); the values so found were consistently very low and subsequently were only determined occasionally. A mean blank value was subtracted in all DOC and corticosterone determinations. The concentration of DOC in the propylene glycol solutions used was determined accurately in every experiment by measuring the optical density at 240 mμ. after dilution with ethanol, and by reference to a calibration curve. The values found were used in calculations of percentage DOC recovered in 'extraction controls' and hydroxylation experiments.

RESULTS

Identification of corticosterone as the product obtained on incubation of DOC with adrenocortical mitochondria

For the experiments to be described in this section DOC was incubated with adrenocortical mitochondria in reaction mixtures containing 0·002 M fumarate. The metabolic product eluted from Celite columns in the 'corticosterone fraction' described in the Experimental section absorbed selectively at 240 mμ. and accounted quantitatively for the amount of DOC which had disappeared. Typical results are shown in Table 2.

In support of this suggestive evidence that the single metabolic product is corticosterone, the material eluted in the 'corticosterone fraction' was run on paper-strip chromatograms using the solvent systems B_1, B_4 and B_5 of Bush (1952). Mixed chromatograms of authentic corticosterone and

metabolic product, metabolic product alone and authentic corticosterone alone were run and developed with the 15% phosphoric acid reagent of Neher & Wettstein (1951). All chromatograms showed spots with a green fluorescence in the ultraviolet, which had run to the same position on the strips. Approximately 50 μg. quantities of metabolic product, authentic DOC and corticosterone were treated with 4 ml. conc. sulphuric acid for 1 hr. at 37° (Zaffaroni, 1950), and absorption curves of the chromogens were determined in a Unicam SP 500 Spectrophotometer. Curves with metabolic product and corticosterone showed maxima at 285, 326, 372 mμ. and between 455 and 465 mμ.

Thus although complete proof has not been obtained, it appears very probable that the predominant or single metabolic product obtained on incubation of adrenocortical mitochondria with DOC is corticosterone.

Citric acid cycle intermediates as activators of DOC 11β-hydroxylation

The results of typical experiments with and without intermediates of the citric acid cycle (called for brevity 'cycle intermediates') (0·002 M) added to the reaction mixture are shown in Fig. 1. It is evident that the presence of certain 'cycle intermediates' will permit the 11β-hydroxylation of DOC. Pyruvate has a negligible effect, whereas citrate, oxaloacetate, L-malate, fumarate, succinate and α-ketoglutarate (α-oxoglutarate) are effective. 0·0005 M-ATP or adenosine diphosphate (ADP) alone are without effect but these substances enhance the hydroxylation obtained with added 'cycle intermediates'. A typical result with succinate supplemented with ATP is included in Fig. 1.

The observed requirements for 'cycle intermediates' in the DOC hydroxylation reaction suggested the possibility of a relationship between steroid hydroxylation and oxidation of the added 'cycle intermediate'. This was investigated by oxygen-consumption measurements made by the usual Warburg technique (Umbreit, 1949). From the results shown in Fig. 1, it may be seen that the 'cycle intermediates' which are oxidized more rapidly are the more effective activators of DOC hydroxylation. ATP which increased the rate of oxidation of 'cycle intermediates' has a corresponding stimulating effect on their ability to activate DOC hydroxylation. Similar results were obtained with ADP. These respiration studies are complicated by the observation that the oxidation of the 'cycle intermediate' is inhibited by the DOC present. In circumstances, under which the inhibition by DOC is sufficiently marked, steroid hydroxylation may be completely suppressed. Such a case is illustrated in Table 3. The use of oxaloacetate as

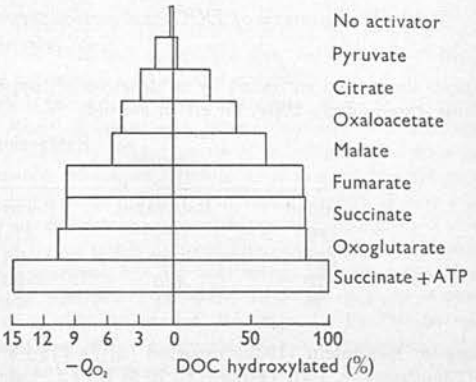


Fig. 1. DOC 11β-hydroxylation and oxygen consumption by adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixture contained 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·04 M potassium phosphate (pH 7·4); 'activators' added as 0·002 M potassium salts (pH 7·4), ATP as 0·0005 M potassium salt (pH 7·4), mitochondria (about 1·4 mg. total N) and 0·04 ml. propylene glycol containing about 500 μg. DOC; total volume, 3 ml.

Table 3. *Influence of DOC on oxidation of tricarboxylic acid cycle intermediates in relation to DOC hydroxylation*

DOC was added as a solution in 0·04 ml. propylene glycol. Propylene glycol alone was added to vessels without DOC.

DOC added (μg.)	DOC recovered (μg.)	DOC recovered (%)	'Cycle intermediate' (0·002 M)	$-Q_{O_2}$
0	—	—	Oxaloacetate	5·6
490	460	94	Oxaloacetate	0·6
0	—	—	L-Malate	10·6
500	5	1	L-Malate	7·8

'activator' normally permits about 40% hydroxylation of DOC (see Fig. 1).

The circumstances giving rise to the differing results reported in Table 3 and Fig. 1 are at present not clear. It is possible that the difference may be attributed to variations in the enzyme preparation or to the age of the adrenal glands used.

In the present case suppression of oxidation has resulted in failure of DOC hydroxylation. When malate was used as 'activator', oxidation was not as markedly suppressed by the added steroid, and hydroxylation of DOC was almost complete.

The effect of magnesium ions on 11β-hydroxylation of DOC

Cohen (1953) observed an increased requirement for magnesium ion when citrate oxidation is used as an energy source for certain synthetic reactions in liver 'homogenates'. As this offered an explanation for the poor activation of DOC hydroxylation by citrate, the effect of varying the magnesium ion concentration was studied. The results shown in

Fig. 2 indicate no increase in DOC hydroxylation in presence of citrate with increased concentration of magnesium ion. In view of the claims of Hayano & Dorfman (1953) it was surprising to observe (Fig. 2) that DOC hydroxylation was diminished but not abolished when added magnesium ion was absent from the reaction mixture using succinate as activator. A 2 g. mitochondrial pellet containing 30 mg. total N was ashed and found to contain less than 10 μg. magnesium oxide by spectrographic analysis. Thus the usual reaction mixtures containing 1·4 mg. mitochondrial total N might be expected to contain less than $3{\cdot}8 \times 10^{-6}$ M magnesium ion.

Effect of different concentrations of fumarate and succinate on 11β-hydroxylation of DOC

If fumarate functions as a hydrogen acceptor for DOC oxidation, according to the reaction fumarate + 2H → succinate, an adequate concentration of fumarate might be expected to permit 11β-hydroxylation to proceed in the absence of oxygen. Similarly, increasing amounts of succinate might be expected to have an inhibitory effect. To test the first possibility, anaerobic experiments with varying concentrations of fumarate were performed in evacuated Thunberg tubes. Results shown in Fig. 3 (*a*) indicate that no hydroxylation occurs in absence of oxygen with concentrations of fumarate up to 0·01 M. The extensive metabolism of DOC in the control tubes opened to the air after evacuation demonstrates that the mitochondrial enzymes involved are undamaged by subjection to low pressures.

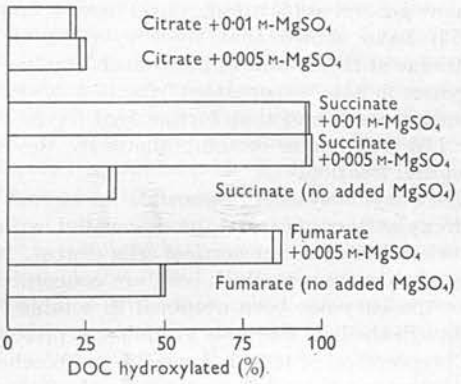


Fig. 2. Effect of Mg^{2+} on 11β-hydroxylation of DOC by adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixture contained 0·095 M-KCl, 0·04 M potassium phosphate (pH 7·4); 'activators' added as 0·002 M potassium salts (pH 7·4), ATP as 0·0005 M potassium salt (pH 7·4), mitochondria (about 1·4 mg. total N), $MgSO_4$ in concentration shown and 0·04 ml. propylene glycol containing about 500 μg. DOC; total volume, 3 ml. Double lines at the ends of bars indicate duplicate values.

The effect of increasing the concentration of succinate (Fig. 3 (*b*)) was to increase DOC metabolism.

The effect of malonate and the activation of DOC hydroxylation by the oxidation of α-oxoglutarate to succinate

The results of experiments described in the previous sections indicate that the concurrent oxidation of certain 'cycle intermediates' will enable 11β-hydroxylation of DOC to proceed. These experiments, however, have not eliminated the possibility of some special requirement for fumarate since this substance will be formed from other intermediates by operation of the citric acid cycle. The formation of fumarate may be prevented by the use of malonate.

Further, it appeared to be of interest to determine whether the 'one step' reaction α-oxoglutarate → succinate could activate 11β-hydroxylation of DOC. The results shown in Fig. 4 indicate that the

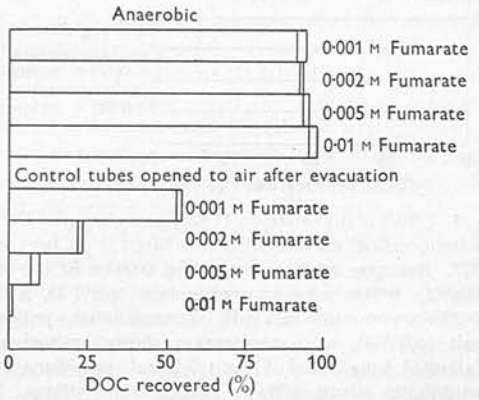


Fig. 3 (*a*). Effect of varying fumarate concentrations on 11β-hydroxylation of DOC.

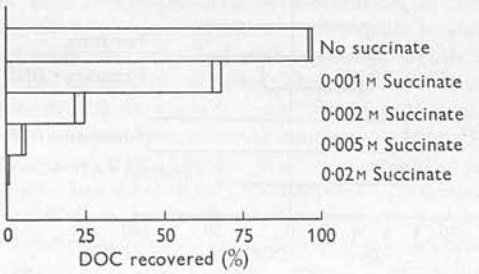


Fig. 3 (*b*). Effect of varying succinate concentration on 11β-hydroxylation of DOC in adrenocortical mitochondria. Reaction mixtures contained 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·04 M potassium phosphate (pH 7·4); 'activators' added as potassium salts (pH 7·4), mitochondria (about 1·4 mg. total N) and 0·04 ml. propylene glycol containing about 500 μg. DOC; total volume, 3 ml. Incubation for 1 hr. at 37°. Double lines at the ends of bars indicate duplicate values.

activation of hydroxylation by 0·002 M succinate is almost completely abolished by 0·01 M malonate. With succinate oxidation blocked in this way it was observed (Fig. 4) that the 'one step' oxidation α-oxoglutarate → succinate enabled DOC hydroxylation to proceed. It thus appears that any specific role for fumarate is excluded.

Effect of 2:4-*dinitrophenol on* 11 *β-hydroxylation of DOC*

Since the 'cycle intermediates' might be involved in processes of oxidative phosphorylation resulting in the activation of DOC for hydroxylation, the effect of 2:4-dinitrophenol on the reaction was investigated. This substance is well known to 'uncouple' oxidative phosphorylation (Loomis & Lipmann, 1948). The addition of 2×10^{-4} M dinitrophenol caused marked inhibition of DOC hydroxylation without having any significant effect on the observed oxygen uptake (Fig. 5).

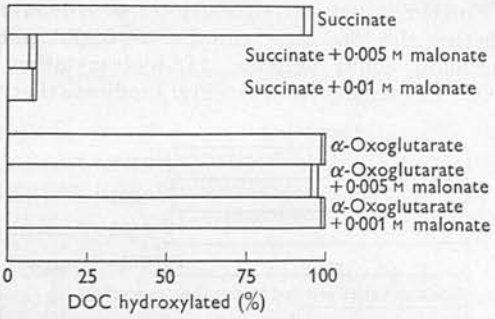


Fig. 4. Effect of malonate on 11β-hydroxylation of DOC by adrenocortical mitochondria incubated in air for 1 hr. at 37°. Reaction mixtures contained 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·04 M potassium phosphate (pH 7·4), ATP as 0·0005 M potassium salt (pH 7·4), malonate as potassium salt (pH 7·4), at concentration shown, mitochondria (about 1·4 mg. total N) and 0·04 ml. propylene glycol containing about 500 μg. DOC; total volume, 3 ml. 'Activators' added as 0·002 M potassium salts (pH 7·4). Double lines at the ends of bars indicate duplicate values.

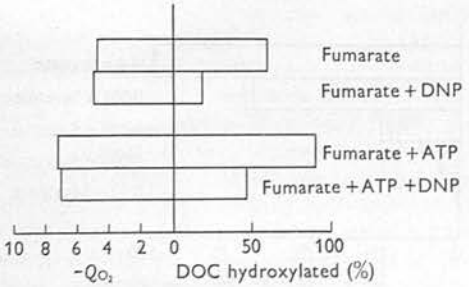


Fig. 5. Effect of 2:4-dinitrophenol on 11β-hydroxylation of DOC and oxygen consumption. Reaction mixture contained 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·04 M potassium phosphate (pH 7·4), fumarate added as 0·002 M potassium salt (pH 7·4), ATP as 0·0005 M potassium salt (pH 7·4), 2:4-dinitrophenol as 0·0002 M potassium salt (pH 7·4), mitochondria (about 1·4 mg. total N) and 0·04 ml. propylene glycol containing about 500 μg. DOC; total volume, 3 ml. Incubation in air for 1 hr. at 37°.

DISCUSSION

In the preliminary investigation of the *in vitro* enzymic hydroxylation of steroids now reported, a method for the determination of DOC has been used which permits the recovery of 96 ± 1 % of this steroid added to preparations of mitochondria. After incubation of DOC with the mitochondria, recoveries of unchanged DOC plus metabolic product as measured by specific absorption at 240 mμ. were of the same order. The percentage recoveries are based upon the actual amount of DOC added to the reaction mixture. These results compare favourably with those obtained by analytical procedures previously reported. For example, Hayano & Dorfman (1953) have made use of a method which permits the recovery of 86 % DOC in unincubated control experiments. After incubation the recovery of unchanged DOC plus metabolic products was 80 ± 5 %. These authors state that 'these figures were taken as indices of 100 in the calculation of the percentage of products formed'. Percentage recovery of metabolic products would thus appear unduly high.

In the present investigation the identity of the metabolic product with corticosterone and the complete absence of other products have not been proved with certainty. Nevertheless, the evidence obtained supports the view that DOC hydroxylation in adrenocortical mitochondria may be largely restricted to the 11β position. This concept may be of more general application, since Plager & Samuels (1953) have shown that hydroxylation of progesterone at the 17 and 21 positions is catalysed by enzymes in the 'supernatant' fraction of adrenal 'homogenates', and that further hydroxylation at the 11β position is brought about by the mitochondrial fraction.

The enzyme system responsible for steroid 11β-hydroxylation appears to be associated with the mitochondria of adrenocortical cells (Sweat, 1951). It is not yet known which cells are concerned nor have the enzymes been obtained in soluble form. Although the best methods available at present for the preparation of morphologically and biochemically intact mitochondria are far from ideal, they are preferable to the procedures frequently used in preparation of adrenal tissue for steroid-hydroxylation studies. Thus, for example, adrenal cell 'homogenates' have been prepared in water or saline by the Waring Blendor (Hayano & Dorfman, 1952; Hayano *et al.* 1951), which when used without speed reduction is known to break up nuclei and mitochondria as well as whole cells (Hogeboom, 1951; Sarkar, Beinert, Fuld & Green, 1952). The

mitochondria in such preparations exposed to water or hypotonic media sustain damage resulting in the release of water-soluble proteins, nucleotides, enzymes and cofactors (Kennedy & Lehninger, 1949; Huennekens & Green, 1950; Schneider, 1949; Berthet, Berthet, Appelmans & Duve, 1951). Incubation of whole 'homogenates' results in extensive destruction of pyridine nucleotides by enzymes released from damaged cell components (Mann & Quastel, 1941). Saline-washed sediments are relatively free from soluble proteins including released nucleotidases. Nevertheless, they contain a mixture of nuclei and mitochondria, intact and damaged by the Waring Blendor and by exposure to saline, and of microsomes agglutinated by the electrolytes present (Schneider & Hogeboom, 1951). The use of such preparations in the study of steroid hydroxylation (Hayano & Dorfman, 1953) must incur the danger of producing artificial steroid metabolic products by interruption of normal metabolic pathways in damaged-cell component particles or by abnormal interaction of enzymes from different cell components. Experiments with washed sediments can give no indication of the location of steroid-hydroxylating enzyme systems in the cell.

The use of mitochondria isolated in sucrose solutions offers several advantages in the preliminary study of steroid hydroxylation. Whole cells, nuclei and soluble proteins may be eliminated so that their effect need not be considered when interpreting results. Contamination by microsomes is not extensive. Schneider (1953) has estimated this to be about 5% of the volume of the mitochondrial sediment. The reduction in the amount of cellular material employed simplifies steroid analysis. Isolated mitochondria retain many dehydrogenases with intact hydrogen- and electron-transporting mechanisms and can catalyse oxidative phosphorylation (Potter, Lyle & Schneider, 1951). Hayano & Dorfman (1953), using washed sediments, were unable to demonstrate 11β-hydroxylation of DOC in absence of added magnesium. The 11β-hydroxylation of DOC observed in experiments now reported in which added magnesium was not required may reflect the intact state of the mitochondria employed. With mitochondria, corticosterone has been obtained as the probable single product of DOC hydroxylation. Hayano & Dorfman (1953) obtained corticosterone and other unidentified products. It remains to be proved that these other products are not artifacts produced by the washed sediment enzyme preparation used by these workers.

Although the use of isolated mitochondria has advantages for the preliminary study of steroid hydroxylation it must not be forgotten that other cell components may have an influence on steroid hydroxylation as it occurs in the intact adrenal cell. 11β-Hydroxylating enzymes may occur in other parts of the cell but may be dependent on mitochondria for energy production or for the provision of terminal electron-transporting mechanisms. These possibilities might be investigated by determining the 11β-hydroxylating capacity of the whole cell as compared with that of mitochondria isolated in maximum yield. Schneider & Hogeboom (1951) and Schneider (1953) have drawn attention to the importance and to the difficulties of obtaining evidence of this nature.

Any explanation of steroid 11β-hydroxylation must account for the role of members of the citric acid cycle in the reaction. The results of the present investigation have confirmed the observations of other workers that the concurrent oxidation of members of the citric acid cycle permits steroid 11β-hydroxylation to proceed. It has now been shown that 11β-hydroxylation is inhibited if succinate oxidation is blocked by malonate. The 'single step' oxidation of α-oxoglutarate to succinate in the malonate-blocked system has been shown to permit 11β-hydroxylation. This observation eliminates the possibility that fumarate has some specific role in the reaction. The failure of fumarate to stimulate 11β-hydroxylation under anaerobic conditions, and the failure of high concentrations of succinate to inhibit 11β-hydroxylation do not support the suggestion that fumarate acts as a hydrogen acceptor. It is very likely that added fumarate enters the citric acid cycle and thereby provides substrates for oxidative reactions coupled with 11β-hydroxylation.

A number of explanations may be offered for the variation in efficiency of different substances as activators of 11β-hydroxylation. Oxaloacetate is not an efficient activator. Low concentrations of this substance inhibit oxidation of succinate (Pardee, Potter & Lyle, 1948) and malate (Elliott, 1941). Less efficient functioning of the citric acid cycle and less rapid 11β-hydroxylation of DOC would thus be expected when oxaloacetate is added as activator as compared with succinate which has no such inhibitory actions. The poor activation obtained with citrate is not explained by the ability of this substance to remove magnesium ions. The observation that citrate does not readily pass through mitochondrial membranes (Schneider, 1953) offers a probable explanation of the poor effect of added citrate. In addition, it is known that the isocitric dehydrogenase activity of isolated mitochondria is low (Hogeboom & Schneider, 1950). These observations indicate a limitation of the *in vitro* technique since mitochondria in the intact cell are not normally confronted with the necessity to utilize high concentrations of individual members of the citric acid cycle.

Kahnt & Wettstein (1951) suggested that oxidative phosphorylation might be involved in

steroid 11β-hydroxylation, but hitherto no evidence has been produced to support this suggestion. It has now been shown that 2:4-dinitrophenol inhibits 11β-hydroxylation of DOC without decreasing the oxidation of the activator. 2:4-Dinitrophenol used in low concentrations, which do not inhibit respiration, is well known to 'uncouple' phosphorylation from oxidation (Loomis & Lipmann, 1948). The role of members of the citric acid cycle in the 11β-hydroxylation of DOC would thus appear to be that of oxidizable substrates for processes of oxidative phosphorylation possibly involved in activation of the DOC before hydroxylation.

In view of the successful linking of 11β-hydroxylation of DOC with the 'single step' oxidation of α-oxoglutarate to succinate, it is interesting to note that Copenhaver & Lardy (1952) observed the highest 'phosphorus esterified/oxygen consumed' ratio for this particular step of the citric acid cycle.

Only those oxidations involving members of the citric acid cycle as substrates have been considered in this report. It is possible, however, that other oxidations coupled with phosphorylation occurring in the adrenal cortex cells are equally capable of 'activating' 11β-hydroxylation of DOC. Since it is probable that in many cases oxidative phosphorylation is associated with hydrogen- and electron-transporting steps of biological oxidation rather than with the primary dehydrogenation (Hunter, 1951), oxidation of reduced pyridine nucleotide coenzymes would be expected to activate 11β-hydroxylation of steroids. Indeed the linking of such hydroxylations with processes of oxidative phosphorylation calls for care when discussing cofactor requirements. It will be necessary to distinguish between cofactors involved in energy-producing reactions and those concerned more directly with steroid hydroxylations. In this connexion the observation of Hayano, Wiener & Lindberg (1953) is of interest. They found that triphosphopyridine nucleotide alone was able to replace the three cofactors—magnesium ions, adenosine triphosphate and diphosphopyridine nucleotide—which were required to regenerate the steroid-hydroxylating activity of enzyme preparations from stored adrenal tissue. A probable interpretation of this result is that triphosphopyridine nucleotide, required for some step of the hydroxylation reaction, is washed out of or destroyed by damaged particles prepared from the stored tissue cells. The enzyme preparation may still retain the ability to synthesize triphosphopyridine nucleotide from diphosphopyridine nucleotide and adenosine triphosphate with magnesium ion as cofactor by reactions described by Kornberg (1951). It remains to be proved however that triphosphopyridine nucleotide acts as a hydrogen acceptor for a reaction in which DOC is directly involved.

It has usually been found necessary to add magnesium ions to reaction mixtures when studying oxidative phosphorylations (Lehninger, 1951). If such reactions are required for activation of 11β-hydroxylation of DOC, the hydroxylation reported above with very low magnesium ion concentrations would be difficult to explain. It is, however, equally difficult to explain the failure to inhibit oxidative phosphorylation by 0·02 M ethylenediaminetetraacetic acid (Lehninger, 1951) since this substance is known to form magnesium complexes with extremly low dissociation constants.

The observation that added adenosine triphosphate alone is unable to activate 11β-hydroxylation of DOC may be related to the similar observation of Kennedy & Lehninger (1948, 1951) in the case of the 'priming' of fatty-acid oxidations. This may find an explanation in the fact that added adenosine triphosphate is less readily available for intramitochondrial reactions, than that generated within the mitochondria (Siekevitz & Potter, 1953).

It has frequently been observed that certain adrenocortical steroids inhibit biological respirations *in vitro*. In particular, Sourkes & Heneage (1952) found that DOC inhibited the oxidation of certain citric acid cycle intermediates by rat-adrenal slices. Hayano & Dorfman (1953) noted the inhibitory effect of increasing concentrations of DOC on the 11β-hydroxylation of this steroid by adrenal-tissue preparations. The significance of these observations in relation to 11β-hydroxylation may be more fully appreciated now that it has been shown that the hydroxylation reaction depends upon concurrent oxidation of suitable substrates, as for instance in the operation of the citric acid cycle, coupled with phosphorylation. The rate of steroid hydroxylation may thus be controlled by the amount of steroid present, the control being effected by the influence of the steroid on oxidations of the citric acid cycle. It is not yet possible to decide whether such a control mechanism has any physiological significance. It points, however, to the possibility that hormones influence enzyme reactions while they themselves are undergoing metabolic changes.

In view of the obvious complexity of the enzymic 11β-hydroxylation of steroids it seems premature to adopt the name '11β-hydroxylase' proposed by Hayano & Dorfman (1953). The term '11β-hydroxylating enzyme system' would be more appropriate at present.

SUMMARY

1. A preliminary investigation of the *in vitro* 11β-hydroxylation of 11-deoxycorticosterone (DOC) by ox-adrenocortical mitochondria has been made.

2. The reaction has been shown to require concurrent oxidative phosphorylation, for which added

citric acid cycle intermediates are oxidizable substrates. The relative efficiency of the intermediates in this role is discussed.

3. Fumarate is not specifically required and the reaction will proceed in the absence of added magnesium ions.

4. The preparation of cell fractions containing steroid 11β-hydroxylating enzymes is discussed. Cofactor requirements are discussed in relation to energy-producing and steroid-hydroxylating reactions.

5. Attention has been drawn to the manner in which certain steroids may influence their own hydroxylation through their effect on oxidative reactions of the citric acid cycle.

It is a pleasure to record our appreciation of the interest which Prof. G. F. Marrian, F.R.S., has shown in this work, and to thank him for his encouragement. The expenses of the work were defrayed in part by a grant to Prof. Marrian from the Medical Research Council. The DOC was a generous gift from Dr C. L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire. British Ropes Ltd., Leith, gave the Nylon pestles for the tissue-disintegrators. The co-operation of the manager and staff of the Offals Department, Wholesale Meat Marketing Association, Edinburgh, in the collection of fresh adrenal glands was of the greatest value. We are indebted to Dr J. W. Minnis for microanalyses, to Dr E. Gilchrist, Clinical Laboratory, Royal Infirmary, Edinburgh, for nitrogen determinations, to Mr A. B. Calder, Edinburgh and East of Scotland College of Agriculture, for magnesium determinations, and to Mr D. W. Davidson for skilled technical assistance.

REFERENCES

Berthet, J., Berthet, L., Appelmans, F. & Duve, C. de, (1951). *Biochem. J.* **50**, 182.
Brendler, H. (1951). *Science*, **114**, 61.
Brownie, A. C., Grant, J. K. & Taylor, W. (1953). *Biochem. J.* **55**, xxii.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Cohen, S. (1953). *J. biol. Chem.* **201**, 93.
Copenhaver, J. H. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.
Elliott, K. A. C. (1941). *Physiol. Rev.* **21**, 267.
Green, D. E., Loomis, W. F. & Auerbach, V. H. (1948). *J. biol. Chem.* **172**, 389.
Harman, J. W. (1950). *Exp. Cell Res.* **1**, 382.
Hayano, M. & Dorfman, R. I. (1952). *Arch. Biochem. Biophys.* **36**, 237.
Hayano, M. & Dorfman, R. I. (1953). *J. biol. Chem.* **201**, 175.
Hayano, M., Dorfman, R. I. & Prins, D. A. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 700.
Hayano, M., Dorfman, R. I. & Yamada, E. Y. (1951). *J. biol. Chem.* **193**, 175.
Hayano, M., Wiener, M. & Lindberg, M. C. (1953). *Fed. Proc.* **12**, 216.
Hogeboom, G. H. (1951). *Fed. Proc.* **10**, 640.
Hogeboom, G. H. & Schneider, W. C. (1950). *J. biol. Chem.* **186**, 417.
Huennekens, F. M. & Green, D. E. (1950). *Arch. Biochem.* **27**, 418.
Hunter, F. E. (1951). In *Phosphorus Metabolism*, **1**, 297, eds. W. D. McElroy and B. Glass. Baltimore: The Johns Hopkins Press.
Kahnt, F. W. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 1790.
Kennedy, E. P. & Lehninger, A. L. (1948). *J. biol. Chem.* **173**, 753.
Kennedy, E. P. & Lehninger, A. L. (1949). *J. biol. Chem.* **179**, 957.
Kennedy, E. P. & Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 361.
Kornberg, A. (1951). In *Phosphorus Metabolism*, **1**, 392, eds. W. D. McElroy and B. Glass. Baltimore: The Johns Hopkins Press.
Lambden, M. P. (1950). *Fed. Proc.* **9**, 193.
Lehninger, A. L. (1951). In *Phosphorus Metabolism*, **1**, 344, eds. W. D. McElroy and B. Glass. Baltimore: The Johns Hopkins Press.
Levy, H., Jeanloz, R. W., Marshall, C. W., Jacobsen, R. P., Hechter, O., Schenker, V. & Pincus, G. (1953). *J. biol. Chem.* **203**, 433.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
McGinty, D. A., Smith, G. N., Wilson, M. L. & Worrel, C. S. (1950). *Science*, **112**, 506.
Mann, J. P. C. & Quastel, J. H. (1941). *Biochem. J.* **35**, 502.
Mattox, V. R. & Kendall, E. C. (1951). *J. biol. Chem.* **188**, 287.
Neher, R. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 2278.
Pardee, A. B., Potter, V. R. & Lyle, G. G. (1948). *J. biol. Chem.* **176**, 1075.
Plager, J. E. & Samuels, L. T. (1953). *Arch. Biochem. Biophys.* **43**, 476.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Potter, V. R. Lyle, G. G. & Schneider, W. C. (1951). *J. biol. Chem.* **190**, 293.
Potter, V. R., Recknagel, R. O. & Hurlbert, B. (1951). *Fed. Proc.* **10**, 646.
Reichstein, T. & Euw, J. von (1938). *Helv. chim. acta*, **21**, 1197.
Sarkar, N. K., Beinert, H., Fuld, M. & Green, D. E. (1952). *Arch. Biochem. Biophys.* **37**, 140.
Savard, K., Green, A. & Lewis, L. A. (1950). *Endocrinology*, **47**, 418.
Schneider, W. C. (1949). In *Manometric Techniques and Tissue Metabolism*, p. 148, by W. W. Umbreit, R. H. Burris and J. F. Stauffer. Minneapolis: Burgess Publishing Co.
Schneider, W. C. (1953). *J. Histochem. Cytochem.* **1**, 212.
Schneider, W. C. & Hogeboom, G. H. (1951). *Cancer Res.* **11**, 1.
Siekevitz, P. & Potter, V. R. (1953). *J. biol. Chem.* **201**, 1.
Sourkes, T. L. & Heneage, P. (1952). *Endocrinology*, **50**, 73.
Steiger, M. & Reichstein, T. (1937). *Helv. chim. acta*, **20**, 1164.
Stern, R. & Bird, L. H. (1949). *Biochem. J.* **44**, 635.
Sweat, M. L. (1951). *J. Amer. chem. Soc.* **73**, 4056.
Taylor, W. (1954). *Biochem. J.* **56**, 463.
Umbreit, W. W. (1949). In *Manometric Techniques and Tissue Metabolism*, p. 1, by W. W. Umbreit, R. H. Burris and J. F. Stauffer. Minneapolis: Burgess Publishing Co.
Zaffaroni, A. (1950). *J. Amer. chem. Soc.* **73**, 3828.

II PUBLICATIONS

[From the Biochemical Journal, Vol. 58, No. 2, pp. 218–225, 1954]

Printed in Great Britain

The *in vitro* Enzymic Hydroxylation of Steroid Hormones

2. Enzymic 11β-hydroxylation of progesterone by ox-adrenocortical mitochondria

By A. C. Brownie, J. K. Grant and D. W. Davidson
Biochemistry Department, University of Edinburgh

(*Received* 20 *April* 1954)

On the basis of experiments in which steroids were perfused through ox-adrenal glands, Hechter *et al.* (1951) were able to present a tentative scheme of adrenocortical hormone biogenesis. Progesterone is postulated as a key intermediate in the scheme, since perfusion of this steroid gave the following products: 17α-hydroxyprogesterone (3–7%), 17α-hydroxycorticosterone (10–12%), corticosterone (1·2–2·4%) and 11β-hydroxyprogesterone (less than 1%). The figures in parenthesis indicate the amounts of steroid recovered. Using ox-adrenal 'homogenates' prepared in potassium chloride solution Hayano & Dorfman (1952) confirmed the ability of the gland to form corticosterone from progesterone. In subsequent experiments using 'residue preparations of twice washed ox-adrenal "homogenates" obtained at 5000 *g*' the same workers (Hayano & Dorfman, 1953) failed to convert progesterone into 11β-hydroxyprogesterone under conditions which permitted the 11β-hydroxylation of 11-deoxycorticosterone (DOC) to take place. They attributed this failure to the absence of a hydroxyl group at the 21-position in the progesterone molecule. The 21-hydroxylating enzyme which Plager & Samuels (1953) have shown to be present in the 'supernatant fraction' of a 'homogenate' centrifuged at 20000 *g* would presumably have been removed on washing the sedimented particles used by Hayano & Dorfman.

In a previous paper (Brownie & Grant, 1954*a*) it was shown that the 11β-hydroxylation of DOC by ox-adrenocortical mitochondria requires concurrent oxidative phosphorylation, for which citric acid cycle intermediates are oxidizable substrates. The observation of Grant & Taylor (1952) that progesterone has a greater inhibitory action than DOC on certain reactions of the tricarboxylic acid cycle in rat-liver mitochondria suggested that similar effects in adrenocortical mitochondria might explain the failure of Hayano & Dorfman (1953) to obtain 11β-hydroxylation of progesterone. Consequently it was decided to study conditions for the 11β-hydroxylation of progesterone by ox-adrenocortical mitochondria with particular reference to the influence of progesterone on reactions contributing to or supporting the hydroxylation reaction. The results of this investigation are now reported.

A preliminary account of this work has been communicated to the Biochemical Society (Brownie & Grant, 1954*b*).

EXPERIMENTAL

Materials and Methods

Melting points were determined on a hot-stage type of apparatus and are corrected. Progesterone was purified by recrystallization from *n*-hexane and aqueous ethanol to give a product melting at 121–121·5°. DOC was prepared from the acetate as described by Brownie & Grant (1954*a*). 11β-Hydroxyprogesterone was a crystalline specimen, m.p. 187–189°. Reichstein & Fuchs (1940) reported m.p. 187–188° for this steroid.

A commercial preparation of the sodium salt of adenosine triphosphate (ATP) (L. Light and Co. Ltd., Slough) was used. Alumina (Peter Spence and Sons Ltd., Widnes) activity II (Brockmann & Schodder, 1941) was used for adsorption chromatography.

In the isolation of ox-adrenocortical mitochondria as described by Brownie & Grant (1954*a*), it has now been found more convenient to use a small Latapie mincer in the cold room at 0° for the preliminary disintegration of the tissue.

Celite 545 and solvents were purified as described by Taylor (1954).

For quantitative experiments, citric acid cycle intermediates and suspensions of mitochondria in the concentrations stated were added to a basal reaction mixture containing 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·0005 M potassium ATP and 0·04 M potassium phosphate (pH 7·4) in a total vol. of 3 ml. Steroids were added in solution in propylene glycol (propane-1:2-diol). Unless otherwise stated, incubations were in air, shaking for 1 hr. at 37°. Each experiment was conducted in four parts as already described (Brownie & Grant, 1954*a*). Incubations were terminated by addition of 15 ml. cold acetone, except when citrate was to be determined, in which case 3 ml. 30% (w/v) trichloroacetic acid was added. After centrifuging, citrate was determined in a portion of the supernatant solution by the method of Taylor (1953).

Oxygen consumption measurements were made by the usual Warburg technique (Umbreit, 1949).

Determination of DOC, progesterone and 11β-hydroxyprogesterone

Steroids were determined by the method described by Taylor (1954) for progesterone with slight modifications. Acetone extracts were not chilled before filtering and benzene–$CHCl_3$ (6:1, v/v) was used for the extraction of

aqueous-acetone residues. The modifications introduced by Brownie & Grant (1954*a*) were used in the method for DOC. Progesterone and 11β-hydroxyprogesterone present together in incubated mixtures were determined in separate portions of the benzene–$CHCl_3$ extract. 11β-Hydroxyprogesterone was isolated by partition chromatography on a Celite column (5 g. Celite 545/4 ml. methanol–water (7:3, v/v)); the solvent systems were benzene–*n*-hexane (3:7, v/v), and methanol–water (7:3, v/v). 11β-Hydroxyprogesterone was eluted in the fraction from 20 to 40 ml. DOC if present would be eluted in the same fraction, but this steroid was not found among the products obtained on incubating washed adrenocortical mitochondria with progesterone.

Steroids eluted from chromatogram columns were determined by measuring their selective absorption at 240 mμ. in ethanol in a Unicam SP 500 spectrophotometer. Amounts of steroid present were found by reference to calibration curves.

Typical results for the recovery of progesterone and 11β-hydroxyprogesterone added to the same incubation mixture containing 200 mg. wet weight mitochondria are shown in Table 1.

'Apparent' steroid recovered in experiments in which no steroids were used was consistently very low (approx. 20 μg.). These values were subtracted in all steroid determinations.

Table 1. *Recovery of progesterone and 11β-hydroxyprogesterone added to reaction mixtures which had been incubated with adrenocortical mitochondria*

Values have been corrected by subtraction of 'apparent' steroid recovered in blank experiments in which no steroids were added.

Added (μmoles)	Recovered (μmoles)	Recovery (%)
	Progesterone	
1·83	1·75	96
1·65	1·56	95
0·744	0·716	96
0·744	0·716	96
	11β-Hydroxyprogesterone	
1·23	1·18	96
1·23	1·17	95
0·712	0·636	89
0·712	0·621	87

RESULTS

Influence of progesterone and DOC on reactions of the tricarboxylic acid cycle supporting 11β-hydroxylation

The influence of progesterone and DOC on citrate metabolism by adrenocortical mitochondria was investigated by measuring the amount of citrate accumulating in reaction mixtures on incubation with pyruvate and L-malate. From the results of a typical experiment shown in Fig. 1 (*a*) it is seen that much less citrate accumulates in the presence of progesterone than with an equivalent amount of DOC. This might be due to a greater inhibition by progesterone of citrate-synthesizing reactions or a greater stimulation of citrate oxidation by progesterone. Direct measurement of the effect of progesterone and DOC on citrate oxidation showed that both steroids inhibited this reaction to about the same extent (Fig. 2). It would thus appear that progesterone exerts a more powerful inhibitory effect on the synthesis of citrate from pyruvate and L-malate than does DOC. The inhibitory action of DOC on citrate synthesis is eliminated and that of progesterone is somewhat decreased if increased amounts of enzyme are used (Fig. 1, *b*, *c*).

Another example of the greater inhibition by progesterone than by DOC is given in Fig. 3 (*a*), which shows the effect of these steroids on the oxygen consumption of mitochondria incubated with succinate.

Concurrent with these measurements of oxygen consumption, rates of metabolism of progesterone and DOC were determined. The results shown in Fig. 3 (*b*) indicate that the added DOC is almost completely metabolized in the first 10 min. of

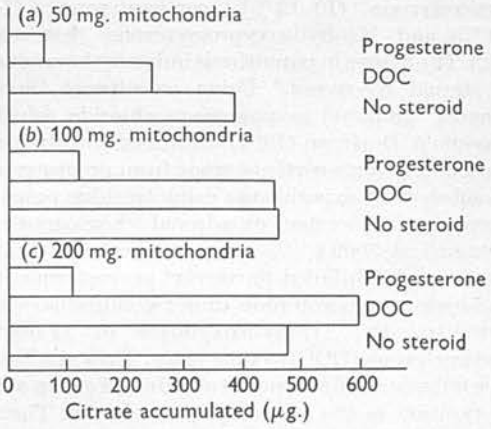


Fig. 1. Effect of progesterone and DOC on citrate accumulation by ox-adrenocortical mitochondria incubated in air for 1 hr. at 37°. Basal reaction mixture supplemented with 0·016 M potassium pyruvate, 0·016 M potassium L-malate and wet weights of mitochondria shown. 500 μg. steroid added in 0·04 ml. propylene glycol.

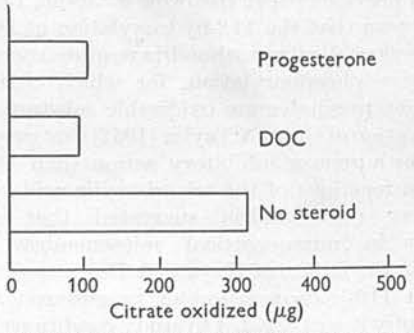


Fig. 2. Effect of progesterone and DOC on citrate oxidation by ox-adrenocortical mitochondria incubated in air for 1 hr. at 37°. Basal reaction mixture supplemented with 0·001 M sodium citrate (576 μg. citric acid/flask) and 100 mg. wet weight mitochondria. 500 μg. steroid added in 0·04 ml. propylene glycol.

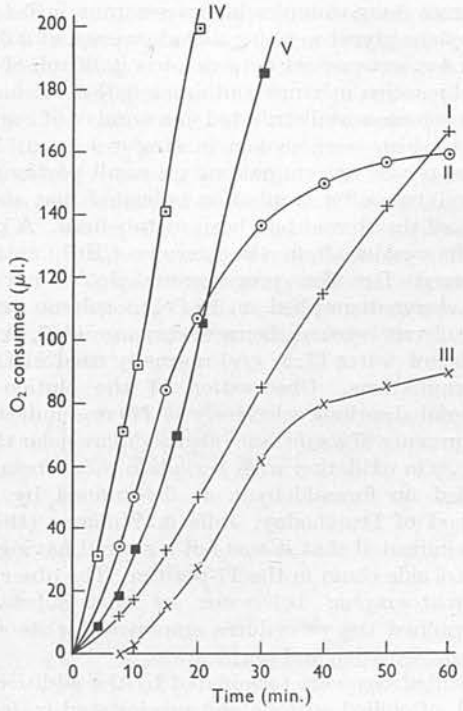


Fig. 3 (*a*). Effect of progesterone and DOC on succinate oxidation by ox-adrenocortical mitochondria. All flasks contained basal reaction mixture. 500 µg. steroids were added in 0·04 ml. propylene glycol. I. 100 mg. wet weight mitochondria, 0·002 M succinate. II. As I plus DOC. III. As I plus progesterone. IV. 200 mg. wet weight mitochondria, 0·01 M succinate. V. As IV plus progesterone.

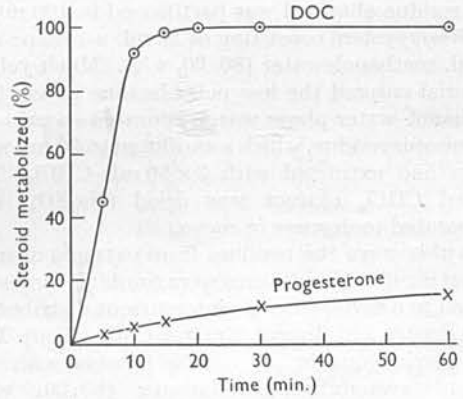


Fig. 3. (*b*). Rates of metabolism of progesterone and DOC by ox-adrenocortical mitochondria. Basal reaction mixture was supplemented with 0·02 M succinate and 100 mg. wet weight mitochondria. 500 µg. steroids were added in 0·04 ml. propylene glycol. Incubations in air at 37°.

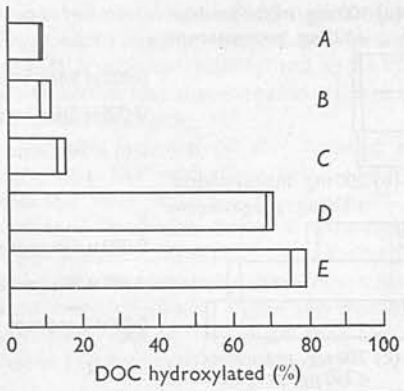


Fig. 4. Effect of pre-incubation of ox-adrenocortical mitochondria on DOC metabolism. 100 mg. wet weight mitochondria were added to the basal reaction mixture. 500 µg. of steroids were added in 0·04 ml. propylene glycol. Incubations were in air at 37°. (Double lines at ends of bars indicate duplicate values.) *A*. Progesterone, DOC and succinate (0·002 M) present from the start, 10 min. incubation. *B*. Progesterone and succinate (0·002 M) present from the start. After 20 min. pre-incubation DOC was added and incubation continued for further 10 min. *C*. After 20 min. pre-incubation, DOC and succinate (0·002 M) were added and incubation continued for further 10 min. *D*. Succinate (0·002 M) present from the start. After 20 min. pre-incubation DOC was added and incubation continued for further 10 min. *E*. DOC and succinate (0·002 M) present from the start, 10 min. incubation.

incubation, a period during which there is a marked stimulation of oxygen consumption. In contrast with this, added progesterone inhibits oxygen consumption and is not itself appreciably metabolized during the first 10 min. This suggests that the adrenocortical mitochondrial enzymes directly or indirectly involved in steroid 11β-hydroxylation may be 'inactivated' during the period in which the mitochondria are not actively respiring. In order to investigate this possibility, mitochondria were pre-incubated under the conditions shown in Fig. 4. The ability of the enzyme preparation to catalyse the 11β-hydroxylation of DOC was then measured. From the results shown in Fig. 4, it is clear that pre-incubation without oxidizable substrate (succinate) or with succinate oxidation inhibited by progesterone, greatly reduces the 11β-hydroxylation of DOC added subsequently. This loss of 11β-hydroxylating activity is not observed if succinate oxidation is uninhibited during the pre-incubation period (bar *D* of Fig. 4).

Determination of conditions suitable for metabolism of progesterone by ox-adrenocortical mitochondria

The results of experiments described above suggested that the inhibition of reactions supporting the 11β-hydroxylation of DOC in ox-adrenocortical

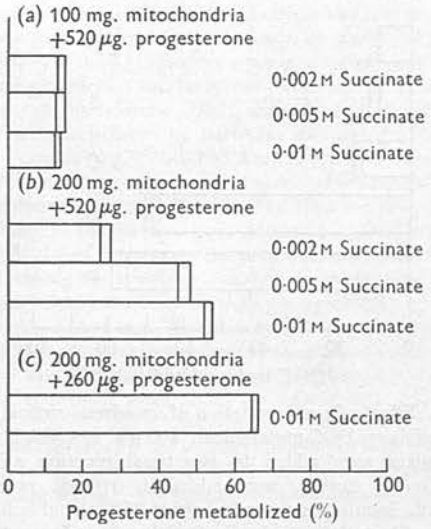


Fig. 5. Effect of varying concentrations of succinate, mitochondria and steroid on progesterone metabolism by ox-adrenocortical mitochondria. Basal reaction mixture supplemented with concentrations of succinate and mitochondria shown. Progesterone in propylene glycol added in the amounts shown. Incubation in air for 1 hr. at 37°. Double lines at ends of bars indicate duplicate values.

mitochondria by progesterone might account for the failure to hydroxylate progesterone itself under these conditions. Conditions were therefore sought which would decrease the inhibitory action of progesterone on the reactions supporting steroid 11β-hydroxylation.

From the results of experiments in which progesterone, succinate and enzyme concentrations were varied (Fig. 5) it appears that incubation of approximately 500 μg. progesterone with 0·01 M succinate and 200 mg. wet weight packed mitochondria (about 6 mg. total N) results in about 50 % metabolism of the progesterone. When the amount of progesterone is reduced to approximately 250 μg. about 65 % is metabolized.

Investigation of the products of metabolism of progesterone by ox-adrenocortical mitochondria

Consideration of the results described in the previous section indicated that the use of 0·01 M succinate and a progesterone:wet packed mitochondria weight ratio of 1:800 in incubation mixtures might be expected to result in 65 % metabolism of the steroid. The difficulty in preparing large amounts of mitochondria from the cortical tissue of fresh ox adrenals limited the amount of steroid which could be incubated at any one time. It appeared that incubation of 45 mg. of progesterone would be convenient for the isolation of the main products of metabolism. Consequently, separate 5 mg. samples of progesterone in 0·4 ml. propylene glycol were incubated on each of 9 days with 4 g. wet packed mitochondria in 60 ml. of the basal reaction mixture containing 0·01 M succinate. This volume was distributed in a number of conical flasks which were shaken in air for 1 hr. at 37°. Progesterone determinations on small portions of the mixture after incubation indicated that about 60 % of the steroid had been metabolized. A part of the residue from the benzene–$CHCl_3$ extract prepared for the progesterone determinations was chromatographed on a Celite column using the solvent system benzene–hexane (3:7, v/v), methanol–water (7:3, v/v) normally used in DOC determinations. Observation of the elution of material absorbing selectively at 240 mμ. indicated the presence of a substance slightly more polar than DOC. On oxidation with periodate this substance yielded no formaldehyde as determined by the method of Daughaday, Jaffe & Williams (1948). This indicated that it was not a steroid having an α-ketol side chain in the 17-position. The observed chromatographic behaviour of this substance determined the procedures employed for its subsequent isolation and purification.

Incubations were terminated by the addition of 5 vol. of chilled acetone and precipitated material was filtered off after standing 2 hr. at −20°. The precipitate was washed with cold acetone. The filtrate and washings were evaporated *in vacuo* to an aqueous residue of about 50 ml. This was diluted with an equal volume of water, saturated with NaCl, and extracted with 3 × 50 ml. ethyl acetate.

The pooled ethyl acetate extracts were washed with 50 ml. 0·2 N-$NaHCO_3$, 50 ml. 0·2 N-HCl and finally with water until the washings were neutral, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The residue obtained was partitioned in 100 ml. of a solvent system consisting of 20 ml. *n*-hexane and 80 ml. methanol–water (80:20, v/v). Much yellow material entered the less polar hexane phase. The methanol–water phase was evaporated *in vacuo* to an aqueous residue, which was diluted to 50 ml. with water and extracted with 3 × 50 ml. $CHCl_3$. The pooled $CHCl_3$ extract was dried (Na_2SO_4) and evaporated to dryness *in vacuo*.

At this stage the residues from extracts of individual incubation mixtures were combined and submitted to a seven-stage countercurrent distribution with double withdrawal (Craig & Craig, 1950). This was carried out in separating funnels with the solvent system methanol–water (80:20, v/v), benzene–*n*-hexane (80:20, v/v), using 50 ml. of each phase. The partition coefficient of progesterone in this system was 0·23 and that of 11β-hydroxyprogesterone 1·6. Fig. 6 shows the theoretical distribution of these two steroids in such an experiment and also the actual distribution of isolated

material absorbing selectively at 240 mμ. Residues obtained on evaporating the contents of funnels 0–6 to dryness *in vacuo* were semi-crystalline. Their combined weight was 12·5 mg. A solution of these combined residues in 3 ml. benzene–*n*-hexane (1:1, v/v) was poured on to a column of about 4 mm. diameter containing 400 mg. Al_2O_3. The chromatogram was developed as shown in Table 2.

The white crystalline material from fractions 7 to 18 was recrystallized from benzene–*n*-hexane (1:1, v/v) to give 8 mg. of crystals melting at 189–191°. On mixing with an authentic specimen of 11β-hydroxyprogesterone, m.p. 187–189°, the m.p. was 187–189°.

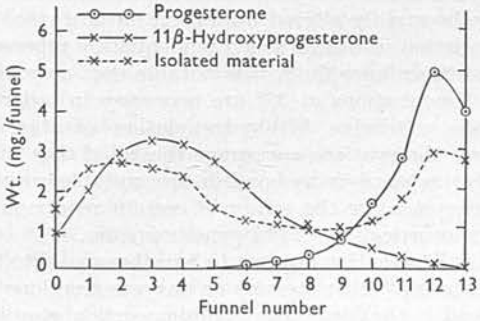


Fig. 6. Theoretical distribution of progesterone and 11β-hydroxyprogesterone and the distribution of isolated material absorbing selectively at 240 mμ. in a seven-stage countercurrent distribution with double withdrawal using the solvent system benzene–*n*-hexane (80:20, v/v) and methanol–water (80:20, v/v). Volume of each phase was 50 ml.

The isolated material and 11β-hydroxyprogesterone (70 μg. each) in stoppered tubes were separately treated with 5 ml. conc. H_2SO_4 for 2 hr. at 17°. Both solutions gave identical absorption spectra over the range 220–520 mμ. (Fig. 7).

The infrared spectra of the isolated material melting at 181–191° and of authentic 11β-hydroxyprogesterone were determined with mulls ground in paraffin in a double-beam Perkin-Elmer 21B spectrophotometer. The positions of all the bands and their relative intensity patterns within each spectrum were identical. From the evidence thus obtained there can be no doubt that the isolated material is 11β-hydroxyprogesterone.

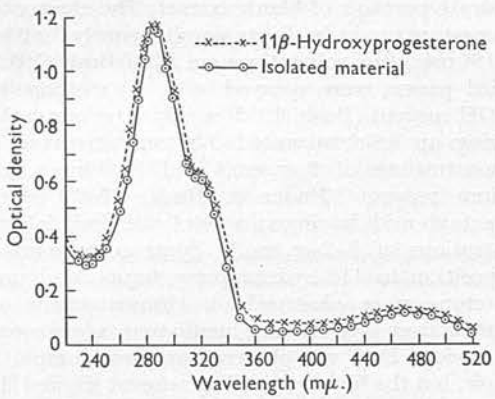


Fig. 7. Absorption spectra of solutions of 11β-hydroxyprogesterone and of isolated crystalline material in concentrated H_2SO_4.

Table 2. *Chromatography on Al_2O_3 of residues from funnels 0–6 in the countercurrent distribution of material obtained on incubation of progesterone with ox-adrenocortical mitochondria*

Each fraction, 3 ml. Solvent ratios, all v/v.

Fraction no.	Solvent	Residue	
		Wt. (mg.)	Description
1–3	Benzene–*n*-hexane (1:1)	—	—
4–6	Benzene–*n*-hexane (3:2)	—	—
7–9	Benzene–*n*-hexane (4:1)	0·5	White crystals
10–12	Benzene	1·4	White crystals
13–15	Benzene–ether (9:1)	6·0	White crystals
16–18	Benzene–ether (4:1)	0·5	White crystals
19–21	Benzene–ether (3:2)	0·5	Amorphous solid

Table 3. *Recovery of progesterone and 11β-hydroxyprogesterone following incubation of progesterone with ox-adrenocortical mitochondria*

Figures in parenthesis indicate the number of results obtained.

Expt.	Progesterone added (μmoles)	Progesterone recovered (μmoles)	11β-Hydroxy-progesterone recovered (μmole)	Total recovery	
				(μmoles)	(%)
1	1·60	1·1±0·05 (8)	0·37±0·04 (8)	1·47±0·1	92±6
2	1·54	0·9±0·06 (8)	0·42±0·07 (8)	1·32±0·13	85±8

The results of quantitative experiments, shown in Table 3, indicate that the 11β-hydroxyprogesterone found may account for the greater part of the progesterone metabolized by ox-adrenocortical mitochondria. Attempts were made to obtain information on the nature of other metabolic products by paper chromatography of the benzene–$CHCl_3$ extract obtained in quantitative experiments. Portions of extract containing about 150 μg. material absorbing selectively at 240 mμ. were employed. For enzyme blanks, similar portions were taken from extracts obtained after incubation of mitochondria without added steroids. For standards, about 150 μg. each of progesterone, 11β-hydroxyprogesterone and DOC were added to separate portions of blank extract. The chromatograms were run in duplicate simultaneously for 2 hr. at 18° using the solvent system B_1 of Bush (1952). Dried papers were sprayed with (*a*) methanolic-NaOH reagent (Bush, 1952) which detects steroids having an αβ-unsaturated 3-ketone structure in concentrations of 2 μg./cm.² and (*b*) blue tetrazolium reagent (Mader & Buck, 1952) which detects steroids having an α-ketol side chain in concentrations of 1–2 μg./cm.². Spots corresponding in position to 11β-hydroxyprogesterone and progesterone were observed on chromatograms of extracts from progesterone incubation experiments. No trace of DOC was observed on these chromatograms, but the blue tetrazolium reagent showed the presence of a trace of material more polar than DOC. This was not observed on chromatograms of progesterone or with the mitochondrial blanks.

DISCUSSION

Hechter (1953*a*) cautiously describes the scheme of adrenocortical-steroid biogenesis which he previously advanced (Hechter *et al.* 1951) as 'a series of deductions based primarily upon the ability of substances to react', and admits the possibility that such *in vitro* reactions may be relatively unimportant in normal metabolic pathways. When evaluating the physiological significance of *in vitro* reactions of adrenocortical steroids and their possible precursors, it is important to bear certain points in mind. The state of enzymes employed and the influence of steroids on enzyme reactions are factors which appear to the present authors to merit special attention.

The unsatisfactory nature of the 'homogenates' and 'washed sediments' used as sources of enzymes for the study of adrenocortical steroid metabolism has already been discussed (Brownie & Grant, 1954*a*). The skilful adaptation of the perfusion technique by Hechter *et al.* (1953) might be expected to preserve adrenocortical enzymes in a natural state, but the results obtained may have been influenced by exposing the perfused steroids to deliberately damaged adrenal tissue.

It is well known that mitochondria, carefully isolated under suitable conditions, contain enzymes and coenzymes which catalyse a wide variety of important reactions including the transfer of hydrogen and electrons to molecular oxygen. In particular, Kielley & Kielley (1951) have shown that such mitochondria catalyse high rates of phosphorylation coupled with oxidation of metabolites of the citric acid cycle. This sequence of reactions has been shown to be necessary for the 11β-hydroxylation of DOC by adrenocortical mitochondria (Brownie & Grant, 1954*a*).

While it can be argued that isolated mitochondria may be rapidly altered from their natural state by incubation in media which inadequately represent the intracellular fluid, it is notable that only very brief incubations at 37° are necessary in order to obtain extensive 11β-hydroxylation of steroids. These observations encourage the belief that carefully prepared mitochondria are suitable sources of enzymes for the study of certain reactions of adrenocortical-steroid biogenesis *in vitro*.

Little attention appears to have been paid to the influence of other steroids on enzyme reactions involved in the biogenesis of adrenocortical steroids. Hechter (1953*b*) has referred briefly to the possible influence of sex hormones on the pattern of adrenocortical steroids synthesized and released from the gland.

Sourkes & Heneage (1952) have observed a stimulation by DOC of succinate oxidation by rat-adrenal halves. Brummel, Halkerston & Reiss (1954) have made the same observation with ox-adrenal slices. This stimulation has now been observed using ox-adrenocortical mitochondria. At the same time the DOC is extensively converted into corticosterone. The oxygen required for this reaction does not however account for the increased oxygen consumption observed.

In contrast with the effect of DOC it has now been found that progesterone inhibits succinate oxidation by adrenocortical mitochondria, and that under such circumstances the 11β-hydroxylation of the steroid is inhibited. It has also been observed in the present work that progesterone has a greater inhibitory action than DOC on reactions involved in the synthesis of citrate from pyruvate and L-malate. It is possible that the inhibitory action of progesterone restricts 'high-energy' phosphate production with resultant inhibition of 11β-hydroxylation of the steroid.

The inhibition by progesterone is most marked during the first 10 min. of incubation. Thereafter oxygen consumption is almost parallel with that in control experiments without added steroid (Fig. 3*a*). No explanation can be offered for this observation.

It appears, however, that enzymes indirectly or directly concerned with 11β-hydroxylation of steroids by adrenocortical mitochondria suffer permanent damage during the first 10 min. of incubation if tricarboxylic acid cycle intermediates are absent, or if their oxidation is inhibited (Fig. 4). This may be explained by the well-known instability under these conditions of enzymes involved in oxidative phosphorylation.

The limited increase in steroid hydroxylation observed on increasing the concentration of enzyme in the case of progesterone as compared with DOC (Fig. 5) suggests that the progesterone effect may not be restricted to an inhibition of respiration. The increased rate of ATP breakdown by rat tissues in the presence of progesterone observed by Jones & Wade (1953) offers an additional explanation for the influence of progesterone on reactions concerned with 11β-hydroxylation.

By suitable adjustment of the concentrations of progesterone, succinate and mitochondria it has now been found possible to decrease the effect of the progesterone on reactions of the tricarboxylic acid cycle sufficiently to permit appreciable 11β-hydroxylation of this steroid.

In the scheme of adrenocortical-steroid biogenesis advanced by Hechter *et al.* (1951), 11β-hydroxyprogesterone is shown as a possible intermediary in the formation of corticosterone or 17α-hydroxycorticosterone from progesterone. The status of 11β-hydroxyprogesterone as such as intermediary has been diminished by the following facts. This steroid has hitherto only been isolated in yields of less than 1 % after perfusion of progesterone through adrenal glands. It has not been found among the products obtained on incubation of adrenocortical tissue preparations with progesterone (Hayano & Dorfman, 1952, 1953). Finally, 11β-hydroxyprogesterone has not been converted into 17α-hydroxycorticosterone or corticosterone in significant amounts (Hechter, 1953*a*). Since it has now been shown to be possible to convert progesterone into 11β-hydroxyprogesterone in good yield by adrenocortical mitochondria it would appear that the status of the latter steroid as an intermediary in the biogenesis of adrenocortical steroids should be reconsidered. The conditions under which attempts have been made to convert 11β-hydroxyprogesterone into corticosterone or 17α-hydroxycorticosterone have not been described by Hechter (1953*a*). It is therefore not at present possible to decide whether these reactions may or may not occur.

The elucidation of the mechanism of adrenocortical-steroid biogenesis is further limited by lack of knowledge of the influence of steroids other than progesterone on the metabolism of their precursors and of the relative affinities of the enzymes concerned for the various steroid substrates which may be present. These problems must form the subjects for future investigations.

SUMMARY

1. Progesterone has been found to be a more powerful inhibitor than 11-deoxycorticosterone (DOC) of certain reactions of the tricarboxylic acid cycle in ox-adrenocortical mitochondria.

2. These inhibitions offer some explanation of the failure of steroid 11β-hydroxylation in presence of progesterone, since this reaction in adrenocortical mitochondria appears to depend upon oxidative phosphorylation for which members of the tricarboxylic acid cycle are oxidizable substrates.

3. Conditions are described under which progesterone may be converted by ox-adrenocortical mitochondria into 11β-hydroxyprogesterone as the main product, and to traces of a more polar product which reduces blue tetrazolium.

4. The choice of suitable enzyme preparations and the influence of steroids on enzymes are discussed in relation to the physiological significance of steroid reactions observed *in vitro*.

It is a pleasure to record our appreciation of the interest which Professor G. F. Marrian, F.R.S., has shown in this work, and to thank him for his encouragement. The expenses of the work were defrayed in part by a grant to Professor Marrian from the Medical Research Council. The DOC acetate was a generous gift from Dr C. L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire. The 11β-hydroxyprogesterone was kindly given by Dr R. Levine, The Upjohn Company, Kalamazoo, Mich., U.S.A. We are indebted to Dr L. J. Bellamy, Chemical Inspectorate, Ministry of Supply, London, for the determination of the infrared spectra. The co-operation of the manager and staff of the Offals Department, Wholesale Meat Marketing Association, Edinburgh, in the collection of fresh adrenal glands was of the greatest value. Mr R. Bayne purified the Celite and the solvents used.

REFERENCES

Brockmann, H. & Schodder, H. (1941). *Ber. dtsch. chem. Ges.* **74**, 73.

Brownie, A. C. & Grant, J. K. (1954*a*). *Biochem. J.* **57**, 255.

Brownie, A. C. & Grant, J. K. (1954*b*). *Biochem. J.* **56**, xxv.

Brummel, E., Halkerston, I. D. K. & Reiss, M. (1954). *J. Endocrin.* **10**, 111.

Bush, I. E. (1952). *Biochem. J.* **50**, 370.

Craig, L. C. & Craig, D. (1950). *Techniques of Organic Chemistry*, vol. 3, ed. Weissberger. New York: Interscience Publishers.

Daughaday, W. H., Jaffe, H. & Williams, R. H. (1948). *J. clin. Endocrin.* **8**, 166.

Grant, J. K. & Taylor, W. (1952). *Biochem. J.* **52**, xxiv.

Hayano, M. & Dorfman, R. I. (1952). *Arch. Biochem. Biophys.* **36**, 237.

Hayano, M. & Dorfman, R. I. (1953). *J. biol. Chem.* **201**, 175.

Hechter, O. (1953*a*). In *Ciba Foundation Colloquia on Endocrinol*, vol. 7, p. 161.

Hechter, O. (1953*b*). *Rev. Canad. Biol.* **12**, 123.
Hechter, O., Jacobsen, R. J., Schenker, V., Levy, H., Jeanloz, R. W., Marshall, C. W. & Pincus, G. (1953). *Endocrinology*, **52**, 679.
Hechter, O., Zaffaroni, A., Jacobsen, R. P., Levy, H., Jeanloz, R. W., Schenker, V. & Pincus, G. (1951). *Recent Progr. Hormone Res.* **6**, 215.
Jones, H. W. jun. & Wade, R. (1953). *Science*, **118**, 103.
Kielley, W. W. & Kielley, R. K. (1951). *J. biol. Chem.* **191**, 485.
Mader, W. J. & Buck, R. R. (1952). *Analyt. Chem.* **24**, 666.
Plager, J. E. & Samuels, L. T. (1953). *Arch. Biochem. Biophys.* **43**, 476.
Reichstein, T. & Fuchs, H. G. (1940). *Helv. chim. acta*, **23**, 684.
Sourkes, T. L. & Heneage, P. (1952). *Endocrinology*, **50**, 73.
Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
Taylor, W. (1954). *Biochem. J.* **56**, 463.
Umbreit, W. W. (1949). In *Manometric Techniques and Tissue Metabolism*, p. 1, by W. W. Umbreit, R. H. Burris & J. F. Stauffer. Minneapolis: Burgess Publishing Co.